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Population genetics of ~~insecticide~~ resistance ~~in the aphid *Myzus persicae*~~

By

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Abstract

The peach-potato aphid *Myzus persicae* (Sulzer) has developed resistance to pyrethroid insecticides as a result of two mutations in the *para*-type sodium channel protein: L1014F (kdr) and M918T (super-kdr). Two allelic discriminating PCR assays were developed that used fluorescent probes to determine precisely the genotype of these mutations in individuals of *M. persicae*. These assays were used alongside existing assays for other resistance mechanisms (MACE and elevated carboxylesterase) to investigate the temporal and spatial incidence of insecticide resistance in *M. persicae*. The kdr mutation and elevated carboxylesterase were found to be widely distributed, being present throughout Europe and in Australia. MACE and super-kdr were widespread in Europe, but were not detected in insects from Australia. A significant deviation from Hardy-Weinberg equilibrium in the populations sampled implied selection against individuals that are homozygous for these resistance mutations. Patterns of distribution in the UK also indicated strong selection against the super-kdr mutation in the absence of insecticide pressure. Significant associations were found between all the different resistance mechanisms, probably promoted by asexual reproduction. The current distribution of the kdr and super-kdr mutations could have arisen by migration from a single source or by independent mutations arising in separate populations. Sequences of intron DNA flanking the mutations showed multiple independent origins of kdr and super-kdr to be the most plausible explanation of these data.

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Finally I would particularly like to thank my wife Angie for moving continents so I could do a PhD at Rothamsted and for her support throughout.

Declaration

The chapters include data from collaborative work, in which I performed all or the major part of the experimental work and the appendix contains co-authored publications which I wrote, and on which I am first author.

James Anstead	Dr Ian Denholm	Prof. James Mallet
Candidate	Supervisor	Supervisor

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Chapter 1.
Introduction and literature review

1.1 Biology of *Myzus persicae* (Sulzer)

Over 250 species of aphids (Hemiptera:Aphididae) are known to feed on crops world-wide and they are one of the most important families of agricultural pests. The genus *Myzus* contains some 55 species (Blackman and Eastop, 2000), of which the peach-potato aphid (*Myzus persicae* Sulzer) (figure 1.1) is the most damaging on a world-wide basis. However other species in this genus are also of economic significance: *M. ascalonicus* Doncaster is a proven vector of some 20 viruses, such as beet mosaic virus (Semal, 1956). *M. cerasi* Fabricius is an important pest of Cherry (*Prunus avium* L.) (Gruppe, 1991) and *M. ornatus* Laing is an occasional virus vector in strawberries (*Fragaria spp.* L.) (Sylvester and Richardson, 1986). Although it is believed to have the same Asian origin as its primary host (peach, *Prunus persicae*) *M. persicae* now has a worldwide distribution (Blackman and Eastop 2000). The importance of *M. persicae* reflects its range, the number of crop hosts it infests and the damage it inflicts through direct feeding and virus transmission.

1.1.1 Pest status

Field crops such as sugar beet (*Beta vulgaris* L.), potatoes (*Solanum tuberosum* L.) and tobacco (*Nicotiana tabacum* L.) suffer significant yield loss from direct feeding damage by *M. persicae*, whose high reproductive rate enables numbers to build up rapidly under favourable environmental conditions. As well as this direct damage, *M. persicae* can transmit a number of important plant viruses such as potato leaf roll virus, barley yellow dwarf virus, cucumber mosaic virus and various sugar-beet viruses (Qi *et al.*, 2004; Nault, 1997). In many systems transmission is a more serious problem than direct damage, and leads to *M. persicae* being sprayed at very low threshold densities. There are also losses in marketability of edible/ornamental crops (Devonshire *et al.*, 1999).

Figure 1.1 A colony of *M. persicae* including alate and apterous individuals

1.1.2 Life Cycle

Like many aphids, *M. persicae* is able to reproduce through both apomictic parthenogenesis and a sexual cycle. It undergoes a single sexual cycle on its primary host; primarily peach (*Prunus persicae* L. including var. *necterina*), and more rarely on Canadian plum (*Prunus nigra* Aiton) and dwarf Russian almond (*Prunus tenella* Batsch), and possibly black cherry (*Prunus serotina* Ehrh) (Blackman and Eastop, 2000). After the sexual cycle *M. persicae* over-winters as an egg. In spring a fundatrix emerges from the egg and feeds and reproduces on the primary host. Changes in the plant's nutritional state and crowding then induce the production of alates (winged forms), which leave the plant to seek a suitable secondary host (Muller *et al.*, 2001). *M. persicae* can utilise a very large number of secondary hosts in over 40 plant families including many economically important plants such as sugar-beet, tobacco, pepper (*Capsicum annum* L.), potato, brassicas (*Brassica spp.*), lettuce (*Lactuca sativa* L.) and sweet potato (*Ipomoea batatas* (L) Lam) (Nebreda *et al.*, 2004; Byamukama *et al.*, 2004; van Emden *et al.*, 1969). Aphids in temperate regions typically show two population peaks separated by a crash during midsummer (Weisser, 2000). A recent review examined the causality of this mid-season crash (Karley *et al.*, 2004) and concluded the main contributory factors were probably depressed performance leading to low birth rate, enhanced emigration and high death rate caused by natural enemies. On secondary hosts it continues to produce numerous apterous and alate progeny until changes in photoperiod and temperature induce the production of specialist male and female gynoparae which migrate back to the primary host to mate. These gynoporous females produce five to fifteen oviparous females that mate with the males and lay cold-hardy eggs (Hardie *et al.*, 1999).

Appropriate *Prunus* hosts are limited in many parts of *M. persicae*'s range and where these hosts are not available, it either does not persist or can reproduce through continuous parthenogenesis (anholocycly) if environmental conditions are favourable (van Emden *et al.*, 1969; Kephalogianni *et al.*, 2002). UK populations have been found to be mostly

anholocyclic or androcyclic (producing sexual males but no sexual females) (Blackman, 1971). In Greece, *M. persicae* has been found to exhibit four different life-cycles existing in sympatry: holocycly, anholocycly, androcycly and intermediate (producing sexual males and females and asexual offspring) (Margaritopoulos *et al.*, 2002). An analysis of *M. persicae* in Scotland using rDNA fingerprinting found evidence for a predominant clone and evidence that many clones persisted from year to year through anholocycly (Fenton *et al.*, 1998). *M. persicae* from South-eastern Australia are a mix of obligate and cyclical parthenogens, the balance between the two life-cycles being determined by the severity of the winter and the availability of peach (Vorburger *et al.*, 2003). Here too, several anholocyclic genotypes were found that were widespread and abundant. This variation in mode of reproduction depends on both the environment and genotype (Blackman, 1974). Even under ideal conditions for sexual reproduction some clonal lineages will not produce sexual morphs or only produce males (Margaritopoulos *et al.*, 2002).

1.1.3 Population genetics

Population structure

Host-adaptation is common within aphids. In the pea aphid (*Acyrthosiphon pisum* Harris), populations on alfalfa and red clover have been characterised as host-races or biotypes (Via, 1999). They are believed to be reproductively isolated as a result of localized host adaptation. Habitat choice lead to assortive mating, providing a barrier to gene flow and subsequent selection lead to the formation of two host races (Caillaud and Via, 2000). There was, however, little genetic differentiation between these biotypes using RFLP markers (Birkle and Douglas, 1999) or mitochondrial DNA sequences (Boulding, 1998). This has lead some to reject the idea of host races in *A. pisum* (Boulding, 1998) and others to conclude the separation into races is very recent (Birkle and Douglas, 1999). In *Schizaphis graminum* (Rondani), sequence data from the mitochondrial COI gene provided evidence that this species is composed of three host-adapted races (Shufran *et al.*, 2000; Shufran, 2003; Anstead *et al.*, 2002). A combination of RAPD-PCR and COII sequences were used by Sunnucks *et al* . (1997b) to separate the aphid

Therioaphis trifolii (Monell) into host-restricted biotypes. Sunnucks *et al* . (1997a) used COI sequences in combination with a microsatellite marker to divide the grain aphid *Sitobion avenae* F. into three distinct races, with little evidence of gene flow between them. Host specialization was found, with one of the lineages found only on wheat, one found only on *Dactylis glomerata* L., and the other found on both. As one of the most polyphagous aphid pest species, with over 400 host plants (van Emden *et al.*, 1969), we might expect similar host-adaptation in *M. persicae.* Weber (1985; 1986) found *M. persicae* performed better on the hosts they were collected from than on alternate hosts. Perhaps the most notable (and controversial) example of host-adaptation concerns the so-called tobacco aphid *M. nicotianae* Blackman. In the 1980's the tobacco aphid was separated from *M. persicae* and given the species name *M. nicotianae* (Blackman, 1987). This species definition was initially based on morphometrics and host use, but was supported by subsequent allozyme studies which showed differences from *M. persicae* (Blackman and Spence, 1992). Two subsequent studies showed that *M. persicae* and *M. nicotianae* had identical sequences in regions flanking amplified carboxylesterases (Field *et al.*, 1994) and in the COII and EF-1$\propto$ genes (Clements *et al.*, 2000a). These results were cited as evidence that the species were not distinct (Clements *et al.*, 2000a). However, given that no variation was found at all, that conclusion is somewhat overstated, as low variation could also indicate a very recent differentiation event. Randomly amplified polymorphic DNA (RAPD's) provided stronger evidence against the designation of *M. nicotianae* as a separate species. The small number of polymorphisms found by Clements *et al.* (2000a) were not correlated with host or geographic origin. A larger amount of RAPD variation was found in a later study (Clements *et al.*, 2000b) which combined RAPDs, GC-MS analysis of cuticular hydrocarbons and host preference tests. This study was unable to distinguish between the species despite considerable variation and the authors concluded there was not a species-level distinction between *M. nicotianae* and *M. persicae.* It should be noted, however, that GC-MS and host preference tests are highly affected by the environment in which aphids

occur. Perhaps most importantly, *M. nicotianae* and *M. persicae* have been shown to interbreed in the laboratory. The former is now generally considered a "tobacco host-race" (Kephalogianni *et al.*, 2002) although the definition is still open to debate, e.g. Dres and Mallet (2002) argue that host races are in fact species, that have more than average gene flow between them.

Other studies have looked at the population structure of *M. persicae* in geographic terms. Guillemaud *et al* . (2003b), Wilson (2002) and Martinez-Torres *et al* . (1997) all observed significant genetic differentiation on various geographical scales and even over relatively small distances (<50km) in Australia (Wilson *et al.*, 2002). Guillemaud *et al* . (2003a) looked at genetic variation at seven microsatellite loci in *M. persicae* in France; they found that genetic differentiation amongst sexual populations was low but significant over distances of 150-200km. They also found lower genetic variation in anholocyclic aphids than holocylic aphids.

Hardy-Weinberg equilibrium

A population in Hardy-Weinberg equilibrium is in a state where the frequency of alleles will remain constant. For this state to exist a number of criteria must be met; the population must be large, there is no migration into or out of the population, individuals must all mate randomly, there is no mutation, all individuals have the same reproductive success and there is no natural selection. Any deviation from Hardy-Weinberg equilibrium may be an indication the population is under selection. Deviations from Hardy-Weinberg equilibrium can be caused by a number of factors; genetic drift, gene flow (e.g. immigration), mutation, non-random mating (e.g. mate choice) or natural selection. Hardy-Weinberg equilibrium has been used to analyse a number of *M. persicae* populations using neutral markers. Populations in Australia were examined using 17 microsatellites and were found to be generally in Hardy-Weinberg equilibrium with a few small deviations (Wilson *et al.*, 2002). Samples collected from France in 2000 were found to be either within Hardy-Weinberg equilibrium or to exhibit a heterozygote deficiency (Guillemaud *et al.*, 2003c), the authors attributed this deficiency to a Wahlund effect (where a heterozygote deficiency is created because the

samples pooled originated from several differentiated populations). In contrast Delmotte *et al* . (2002) found significant heterozygous excess in asexual *R. padi* populations; this heterozygosity was higher in anholocyclic than holocyclic insects. The authors believed this to be due to either an ancient loss of sexuality in the anholocyclic clones or that these clones were formed by hybridisation.

1.2 Insecticide resistance in *M. persicae*

The chemical control of insect pests has become an integral part of crop production and disease prevention during the last 50 years. One of the consequences of widespread insecticide spraying has been the development of resistance to these chemicals. At the last count over 500 species of arthropods were resistant to one or more chemical control agent (Georghiou, 1990). For a number of pests the development of multiple mechanisms of resistance to different classes of insecticides has made control extremely difficult and expensive. There are several important consequences of the development of resistance. Firstly there is a constant need to develop new insecticides as old ones become ineffective. This is expensive and has added significantly to the cost of control. Secondly there has been a tendency to increase doses to overcome resistance, this increases costs, compounds environmental problems and may lead to faster resistance development.

1.2.1 Resistance mechanisms

Resistance mechanisms can be broadly divided into two categories, ones based on target-site modification or ones based on enhanced detoxification of insecticide.

Target-site resistance is characterised by specific point mutations in insecticide target proteins which cause them to become insensitive to the insecticide. The three major types of target-site resistance are; kdr (knockdown resistance to pyrethroids) involving the voltage-gated sodium channel, MACE (modified acetylcholinesterase), and *Rdl* (resistance to dieldrin) involving the $GABA_A$ receptor. These mutations are found in a broad range of insects (kdr will be discussed in more detail later). *Rdl*

mutations conferring resistance to organochlorine insecticides (especially cyclodienes) have been found in *Drosophila melanogaster* Meigen (ffrench-Constant *et al.*, 1993b), *D. simulans* Sturtevant (ffrench-Constant *et al.*, 1993a; ffrench-Constant *et al.*, 1993b) *Musca domestica* L., *Periplaneta americana* L. , *Tribolium castaneum* Herbst (Thompson *et al.*, 1993b), *Aedes aegypti* L., (Thompson *et al.*, 1993a), *Anopheles stephensi* (Liston) (Andreasen and ffrench-Constant, 2002), *Hypothenemus hampei* (Ferrari) (ffrench-Constant *et al.*, 1994), *Blattella germanica* L. (Kaku and Matsumura, 1994), *Bemisia tabaci* Gennadius (Anthony *et al.*, 1995a), *Myzus persicae* (Anthony *et al.*, 1998) and *Ctenocephalides felis* Bouche (Bass *et al.*, 2004b). In the great majority of cases the same mutation (alanine302 to serine) has arisen independently in the GABA receptor gene. MACE (conferring resistance to organophosphates and carbamates) has also been found in a number of insect species including; *D. melanogaster* (Mutero *et al.*, 1994), *Musca domestica* (Walsh *et al.*, 2001), *Myzus persicae* (Nabeshima *et al.*, 2003; Andrews *et al.*, 2002), *Aphis gossypii (Andrews et al., 2004)*, *Culex pipiens pipiens* L., *C. p. quinquefasciatus* Say and *C. tritaeniorhynchus* Giles, *Anopheles nigerrimus* Giles, *An. atroparvus* Van Thiel, *An. sacharovi* Favre and *An. gambiae* Giles (N'Guessan *et al.*, 2003; Bisset *et al.*, 1990; Hemingway *et al.*, 1986; Villani and Hemingway, 1987; Hemingway *et al.*, 1985; Hemingway, 1983; Hemingway, 1982)

Detoxification mechanisms are characterised by the up-regulation of enzyme production, or a qualitative change in a detoxifying enzyme. The enzymes involved include cytochrome P450 dependent monooxygenases or non-specific esterases such as carboxylesterases. There are about 100 P450 genes within the insect genome, carrying out a broad range of functions (Feyereisen, 1999), one of which is the metabolism of xenobiotics. A large number of different P450's have been implicated in insecticide resistance (for a thorough review see Feyereisen (1999)) though in Drosophila a single P450 was found to be responsible for resistance to DDT, imidacloprid and malathion (Le Goff *et al.*, 2003). Esterases also cause resistance in a diverse variety of insects such as the California red scale *Aonidiella aurantii* (Maskell) (Grafton-Cardwell *et al.*, 2004), Colorado potato beetle

Leptinotarsa decemlineata (Say) (Anspaugh *et al.*, 1995), *Culex* mosquitoes (Mouches *et al.*, 1987) and aphids such as *M. persicae* and the damson-hop aphid *Phorodon humuli* Shrank (Lewis and Madge, 1984; Devonshire and Sawicki, 1979).

Chemical control is widely used against *M. persicae* and resistance has developed to a number of insecticide groups. Resistance was first noticed in the USA during the 1950's and was confirmed in 1963 (Georghiou, 1972). The first resistance mechanism identified in *M. persicae* was the over-production of carboxylesterases, which sequester and detoxify insecticides, giving high resistance to organophosphates and limited resistance to carbamates and pyrethroids. This resistance was first demonstrated by showing that resistant clones showed increased hydrolysis of the model ester naphthyl acetate (Needham and Sawicki, 1971). It was subsequently shown that this over-production was the result of gene amplification (Devonshire and Sawicki, 1979) and that there were two similar genes (E4 and FE4) either of which could be amplified (Field *et al.*, 1993). Only very rarely are both genes amplified together (Blackman *et al.*, 1999).

Mutations of two target-sites can also confer insecticide resistance in *M. persicae*. The enzyme acetylcholinesterase is the principal target of carbamates such as pirimicarb and organophosphates. Individuals of *M. persicae* with modified acetylcholinesterase (MACE) show high levels of resistance to dimethyl carbamates such as pirimicarb and triazamate (Moores *et al.*, 1994) and are now widespread in Europe and the UK (Foster *et al.*, 1998). The mutation responsible for the MACE phenotype is a single amino acid substitution of Ser431Phe in the *M. persicae* acetylcholinesterase2 protein (Nabeshima *et al.*, 2003; Andrews *et al.*, 2002).

The voltage-gated sodium channel is the target site of DDT and pyrethroid insecticides. Mutations in the sodium channel genes are responsible for so-called knockdown resistance.

1.2.2 Knockdown resistance

Synthetic pyrethroids were developed from insecticidal compounds found in extracts of chrysanthemum (pyrethrum). They have been used since the first

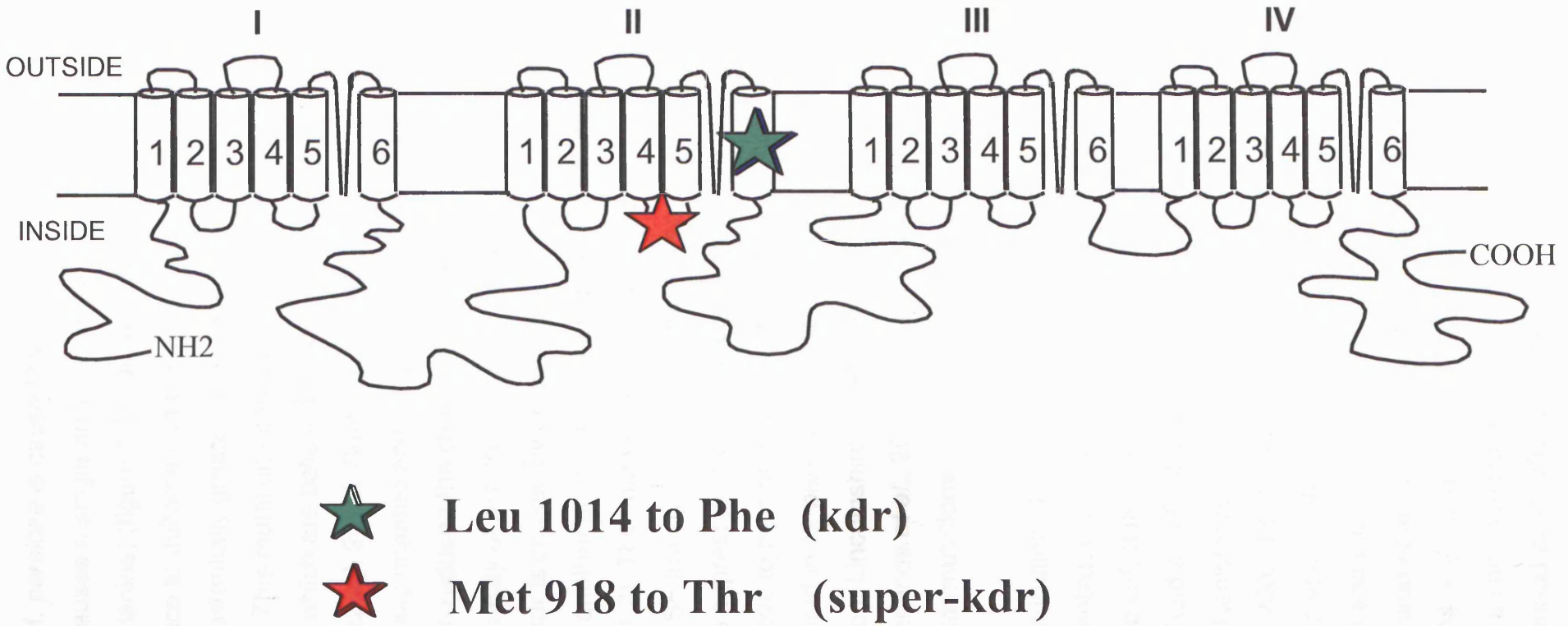
century BC in China for their insecticidal properties (Lhoste, 1964). The first synthetic pyrethroid (allethrin) was developed in the late 1940's (Schechter *et al.*, 1949) but because of poor photo-stability the use of pyrethroids was restricted to household and veterinary uses (Leahey, 1979). The first photostable pyrethroid was permethrin (Elliott *et al.*, 1973), its enhanced stability greatly extended the potential applications of pyrethroids.

The insect voltage-gated sodium channel is a transmembrane protein complex that forms a voltage-gated pore. The main α-subunit is a large glycoprotein composed of four homologous repeated domains (figure 1.2). These are arranged in a circular pattern in the lipid bilayer to form the pore. Each domain consists of six transmembrane segments connected by intracellular and extracellular loops. During the transmission of a nerve impulse, membrane depolarisation causes a conformational change in the protein, opening the pore and increasing sodium permeability, the pore spontaneously closes after a few milliseconds, becoming inactivated. The pore then returns to a resting (closed) state until it is reactivated by a further depolarisation. Pyrethroids selectively alter the kinetics of the sodium channel, in particular slowing down its inactivation, prolonging the sodium influx during excitation (Georghiou, 1972; Soderlund and Bloomquist, 1990; Vijverberg and Vandenbercken, 1990; Narahashi, 1992; Bloomquist, 1996). This prolonged influx of sodium results in further depolarisation of the membrane and repetitive firing of the nerve (Beeman, 1982; Miller and Adams, 1982). Affected insects quickly become paralysed and die. Initial data indicated kdr mutations behave as a largely recessive trait,(Dong, 1997; Park *et al.*, 1997; Schuler *et al.*, 1998; Williamson *et al.*, 1996). More detailed, later studies show the L1014F mutation is incompletely recessive in *M. persicae* (Foster *et al., 2002a)*, *Cydia pomonella* (L.) (D = -0.199) (Bouvier *et al.*, 2001) and *Helicoverpa armigera* (Tan and McCaffery, 1999) (D = -0.66). Modified sodium channels conferring knockdown resistance (kdr) or super-knockdown resistance (super-kdr) to pyrethroids have been documented from a large number of species (Table 1.1).

Table 1.1 Reported resistance mutations in the insect sodium channel

Common name	Scientific name	Mutation	Reference
Housefly	*Musca domestica*	L1014F	(Williamson *et al.*, 1993)
		M918T	(Williamson *et al.*, 1996)
Human headlouse	*Pediculus capitis*	T929I	(Lee *et al.*, 2000)
		L932F	
		M815I	(Lee *et al.*, 2003)
German cockroach	*Blatella germanica*	L1014F	(Dong, 1997)
		E435K	(Liu *et al.*, 2000)
		C785R	
		D59G	
		P1999L	
Horn fly	*Haematobia irritans*	L1014F	(Guerrero *et al.*, 1997)
		M918T	
Diamond back moth	*Plutella xylostella*	L1014F	(Schuler *et al.*, 1998)
		T929I	
Mosquito	*Anopheles gambiae*	L1014F	(Martinez-Torres *et al.*,
		L1014S	1998), (Ranson *et al.*, 2000)
Mosquito	*Anopheles arabiensis*	L1014F	(Diabate *et al.*, 2004)
Mosquito	*Anopheles stephensi*	L1014F	(Enayati *et al.*, 2003)
Mosquito	*Culex pipens*	L1014F	(Martinez-Torres *et al.*,
		L1014S	1999a)
Cattle tick	*Boophilus microplus*	F1538I	(He *et al.*, 1999)
Mosquito	*Anopheles sacharovi*	L1014F	(Luleyap *et al.*, 2002)
		L1014S	
Colorado potato beetle	*Leptinotarsa decemlineata*	L1014F	(Lee *et al.*, 1999)
Tobacco budworm	*Heliothis virescens*	L1014H	(Park and Taylor, 1997;
		V410M	Park *et al.*, 1997), (Head
		D1549V	*et al.*, 1998)
		E1533G	
Vinegar fly	*Drosophila melanogaster*	I253N	(Pittendrigh *et al.*, 1997)
		A1410V	
		A1494V	
		M1524I	
Tobacco whitefly	*Bemisia tabaci*	M918V	(Morin *et al.*, 2002)
		L925I	
Cotton bollworm	*Helicoverpa armigera*	D1549V	(Head *et al.*, 1998)
		E1533G	
Cat flea	*Ctenocephalides felis*	L1014F	(Bass *et al.*, 2004a)
		T929V	

Figure 1.2 Schematic representation of the α-subunit of the voltage-gated insect sodium channel protein, indicating the location of resistance mutations in *M. persicae.* (Adapted from Williamson et al. 1996)



Kdr in *M. persicae* is caused by a single nucleotide change (TTC-to-CTC) which causes a single amino-acid substitution L1014F (leucine-to-phenylalanine) (figure 1.2). On its own this mutation confers 35-fold resistance to deltamethrin but in conjunction with high carboxylesterase can give substantially greater resistance to pyrethroids (Martinez-Torres *et al.*, 1999b). This mutation occurs within one of the lipophilic S6 transmembrane regions which are believed to surround the sodium channel pore (Lipkind and Fozzard, 2000). Smith *et al.* (1997) and Vais *et al.* (2000) expressed specifically mutated and wildtype sodium channels and showed that this mutation reduced the channels' sensitivity to pyrethroids and increased the rate of decay of pyrethroid-induced sodium tail currents.
Super-kdr is caused by an additional nucleotide change (ATG-to-CTG) which causes another amino-acid substitution M918T (methionine-to-threonine) (figure 1.2). This mutation occurs within the intracellular linker between the S4 and S5 transmembrane regions. When co-expressed with kdr in *Xenopus* oocytes expression systems the sodium channels produced were completely insensitive to pyrethroids (Lee *et al.*, 1999). Super-kdr was initially discovered in *M. persicae* in a clone designated 2169G, collected in October 1997 from Lincolnshire. It exhibited >1000 fold resistance to pyrethroids (Eleftherianos *et al.*, 2002) and was heterozygous for both the kdr and super-kdr mutations.

The distribution of insecticide resistance in *M. persicae* is reasonably well documented. Carbamate resistance (caused by MACE) has been recorded from the UK, Italy, Greece, Holland, Argentina and Japan (Foster *et al.*, 1998; Field and Foster, 2002; Mazzoni and Cravedi, 2002; Field *et al.*, 1997). Resistance to pyrethroids has been recorded in the UK (Field and Foster, 2002; Fenton *et al.*, 2005; Foster *et al.*, 1998; Field *et al.*, 1997; Anstead *et al.*, 2004), in Italy, France, Spain, Greece, Germany, Holland (Nauen and Elbert, 2003; Field *et al.*, 1997; Mazzoni and Cravedi, 2002; Guillemaud *et al.*, 2003b), the USA, Japan and Chile (Fuentes-Contreras *et al.*, 2004; Field *et al.*, 1997). This resistance was shown to be caused by elevated carboxylesterases and the kdr mutation in all these populations (Guillemaud *et al.*, 2003a; Fenton *et al.*, 2005; Anstead *et al.*, 2004; Foster

et al., 1998; Field and Foster, 2002) except those from Chile in which it was due to elevated levels of carboxylesterases alone (Fuentes-Contreras *et al.*, 2004).

Australian populations have been found to contain the 1,3 autosomal translocation associated with elevated E4 carboxylesterase (Wilson *et al.*, 2002), indicating this resistance mechanism is probably present. Most of these results however are based on just a few individuals and the frequency of these mechanisms is impossible to determine.

Prior to this study only a single clone (2169G) had been found with the M918T super-kdr mutation(Eleftherianos *et al.*, 2002). Many *M. persicae* have been found to contain multiple resistance mechanisms; for example, individuals collected from the UK and Greece in 1997 had R3 levels of carboxylesterase, the MACE mechanism and were homozygous resistant at the kdr locus (Foster *et al.*, 2003b).

1.2.3 Resistance Monitoring

The value of monitoring for insecticide resistance is undeniable. Large amounts of money are spent on chemical insect control each year and a substantial (though un-quantified) proportion is wasted by spraying resistant pests. Insecticide resistance is also the perfect model to study the evolution of adaptive genes, as the phenotype and genotype are often well understood and selection occurs across a very short time-frame. A number of different techniques have been used to monitor for insecticide resistance. The technique utilised depends on the type of resistance suspected to be present and to what extent the biochemical and/or molecular basis of that resistance has been resolved.

Elevated carboxylesterases

Elevated E4/FE4 carboxylesterases contribute to resistance to pyrethroids as well as to organophosphates and carbamates. In *M. persicae* a number of techniques have been used to diagnose this mechanism. The total amount of esterase within an individual was found to be a good indicator of the over-production of these carboxylesterases, but the most accurate method was an immunoassay using an antiserum specific to the E4/FE4 enzymes conferring resistance (Devonshire *et al.*, 1986; Moores *et al.*, 1994). This

technique gives a colorimetric change when aphid homogenate is applied to a 96 well plate coated with IgG (immunoglobulin G). Quantitative-competitive PCR has also been used to estimate gene copy number (Field *et al.*, 1999) However, as amplified genes may be switched off by methylation (Field, 2000), this may not be a good indication of the resistance phenotype.

Target-site resistance

The MACE mechanism in *M. persicae* is most commonly diagnosed using a kinetic microplate assay measuring AchE activity in the presence and absence of insecticide inhibitors (Moores *et al.*, 1994). As kdr is not amenable to biochemical assays all techniques have focused on either bioassays or detection of the point mutations responsible for the resistance. A diagnostic dose bioassay using DDT was one of the first techniques for detecting kdr (Field *et al.*, 1997). This was followed by a number of molecular techniques which didn't require rearing live insects, including DNA sequencing, single strand conformation polymorphism for kdr (SSCP) (Eleftherianos *et al.*, 2003) and PCR amplification of specific alleles for kdr (PASA) (Guillemaud *et al.*, 2003a). All are relatively time-consuming and the development of a more rapid PCR-based diagnostic was an important objective of the current project.

1.3 Scope and objectives of project

There were 5 main objectives to this project

1. Develop and test a high-throughput detection system for the kdr and super-kdr mutations. Ideally this would not require the insects to be reared and would run alongside existing tests for elevated carboxylesterase and MACE, with all three tests being run on a single *M. persicae* individual.
2. Confirm that the super-kdr mutation is associated with high levels of insecticide resistance.
3. Determine the spatial and temporal spread of kdr mutations, primarily within the UK but also in other countries if samples were available.
4. Examine the relationship between the three different resistance mechanisms to look for positive or negative associations between them.
5. Test the null hypothesis that the kdr and super-kdr mutations had single world-wide origins.

Chapter 2.
High-throughput detection of knockdown resistance in *Myzus persicae* using allelic discriminating quantitative PCR

Abstract

The peach-potato aphid *Myzus persicae* (Sulzer) has developed resistance to pyrethroid insecticides as a result of a mechanism conferring reduced nervous system sensitivity, termed knockdown resistance (kdr). This reduced sensitivity is caused by two mutations, L1014F (kdr) and M918T (super-kdr), in the para-type voltage gated sodium channel. A diagnostic dose bioassay was developed to detect knockdown resistance and provide preliminary information on the genotype present. Two allelic discrimination PCR assays were also developed to determine precisely the genotypes of the two mutations (L1014F and M918T) in individual *M. persicae* using fluorescent Taqman® MGB probes. In combination with assays for elevated carboxylesterase levels and modified acetylcholinesterase (MACE) this suite of assays allows for rapid high-throughput diagnosis, in individual aphids, of the three main resistance mechanisms of practical importance in the UK.

2.1 Introduction

One of the main aims of this project was to develop a high-throughput detection system for diagnosing the kdr and super-kdr mutations in *M. persicae*. There were a number of important criteria that had to be fulfilled by a successful detection system. Firstly it had to run alongside existing tests for elevated carboxylesterase and MACE. This meant the test had to be sensitive enough that it could be run on a fraction of an individual so all three tests could be run on the same aphid. Rearing aphids for live testing (e.g. bioassays) is time-consuming and labour- intensive so ideally the detection system would work equally well on aphids preserved by freezing or in alcohol. The test had to be accurate since resistance alleles are often found at relatively low frequency within a population. It also needed to differentiate homozygotes and heterozygotes for the application of population genetics. False positives and false negatives need to be avoided; false positives would lead to the erroneous diagnosis of resistance and ring unnecessary alarm-bells whereas false negatives could mask the presence of resistance. If the test was to be eventually used for screening field samples to inform decisions on control methods it also needed to be fast and have a high-throughput. Ideally results would need to be generated within one or two days of the samples being received and several hundred samples could conceivably need to be tested in one day. Such tests have already been developed for carboxylesterase levels and MACE (Devonshire *et al.*, 1986; Moores *et al.*, 1994). These tests run in a 96 well microplate format and can provide a resistance profile the day that samples are collected (see chapter one for details).

Given these requirements it was clear that a DNA based method was needed. A number of approaches were considered initially and discounted. DNA sequencing (Eleftherianos *et al.*, 2002) had been used for some time to detect kdr mutations but it is not particularly high-throughput and it is sometimes difficult to separate heterozygotes and homozygotes. Single strand conformation polymorphism (SSCP) had also been used with some success to screen for kdr, but failed to produce consistent results for super-kdr. PCR-based allelic discrimination assays using fluorescent dye-labelled

probes have already been developed for high-throughput screens to detect mutations in other systems and provide fast and reliable results (Ginzinger, 2002; Livak, 1999) and quantitative assays using the same system have recently been developed to determine resistance levels in strobilurin resistant fungi (Fraaije *et al.*, 2002). In this chapter two assays are described, a simple low cost screen for the kdr and super-kdr phenotypes and a high-throughput allelic discrimination assay, for L1014F and M918T, that can be run in conjunction with MACE and carboxylesterase tests developed previously, and applied to the same individual aphids.

2.2 Materials and Methods

Aphids were collected from a number of UK sites. Live samples were shipped in Perspex "Blackman" boxes (Blackman, 1971) on potato or Chinese cabbage leaves. Clonal lineages were started from a single adult from these samples and reared on Chinese cabbage (*Brassica napus* var chinensis cv "Wong-Bok) leaves in leaf boxes in controlled environment rooms at 20 ± 1°C under 16:8 hours (light:dark).

A diagnostic dose bioassay was developed as a straightforward means of screening samples for the presence of kdr mutations. The assay was validated using laboratory clones of known genetic composition and then applied to batches of 10 apterous adults from 25 field collected clones. The standard clones used were 800F (wildtype), 1316A (heterozygous for L1014F, homozygous for M918), 794J (homozygous for F1014, homozygous for M918) and 2169G (heterozygous for L1014F and M918T) (Martinez-Torres *et al.*, 1999b; Eleftherianos *et al.*, 2002). Aphids for testing were transferred from colonies of a clonal lineage on to a 3cm Chinese cabbage leaf disc that had been placed upside down on a bed of agar (1.5%) in plastic pots. Five pots each containing ten individual apterous aphids were set up and tested for each clone. A 0.25 µl, topically applied dose of 100ppm deltamethrin (a pyrethroid) was used. Bioassays were assessed after 72 hours and aphids were scored as alive or dead.

For genotypic testing single aphids were homogenised in 50µl of PBS/Tween (Phosphate buffer, 0.02M, pH 7.0 containing 0.05 v/v Tween 20) in the wells of a microtitre plate using a multihomogenizer (french-Constant and Devonshire, 1987). 1µl was used for carboxylesterase testing, 5µl was removed for the extraction of genomic DNA and the remainder was used to test for the MACE phenotype.

Genomic DNA was extracted from the homogenized samples using Dynazol® (Helena biosciences) at a fifth scale. Genomic DNA from each aphid was re-suspended in 10 µl of TE buffer.

For each mutation site (kdr=L1014F and super-kdr=M918T) two primers and two minor groove binding (MGB) probes (Applied Biosystems) were designed using Primer Express™ Version 1.5 (primers: kdr1=ccattcttcttggctacgttgtc, kdr2=ccgagtagtacatatttatcattcat, skdr1=cgtggcccacactgaatct, skdr2=ttatgcacaagacaaacgttaggtta. Probes: kdr-s=6FAM- accacgaggttacc, kdr-r=VIC-ataccacgaagttacc skdr-s=6FAM-cgacccattatggatat, skdr-r=VIC-ataccacgaagttacc). Each probe consists of an oligonucleotide, a 5' reporter dye, a 3' nonfluorescent quencher (TAMRA) and a minor groove binder at the 3' end. In both probe sets 6-FAM is linked to the 5' end of the probe for the detection of the wildtype allele and VIC is linked to the 5' end of the probe for the detection of the resistant allele. The minor groove binder provides more accurate allelic discrimination by increasing the T_m between matched and mis-matched probes (Afonina *et al.*, 1997).

Short PCR fragments containing the mutation sites were amplified separately using the flanking primers kdr1 and 2, skdr 1 and 2. The primer and probe concentrations were optimised to give the best discrimination between the annealing of the two probes in each assay. The assays were run in 96 well plates with 25µl reaction volumes. Each reaction contained 12.5 µl of Taqman ® universal PCR master mix (Applied Biosystems), 7.5µM of each primer, 5µM of each probe and 1µl of genomic DNA made up to 25µl with filter sterilised water. Each plate contained triplicates of wildtype controls, resistant type controls and no template controls. DNA from 3 individuals of each strain was extracted separately and ran them in individual

wells. The PCR was performed on an ABI Prism ® 7700 (heated lid) running the following thermal cycle (50°C for 2 min, 95°C for 10 min, followed by 35 cycles of 92°C for 15 seconds, 60°C for 1 min). The PCR was automatically analysed in real-time and at the end point.

DNA sequencing was used to confirm the accuracy of the allelic discrimination assay using control insects and 12 randomly selected clones (shown in Figure 2.3). A 493bp fragment from the domain II region of the sodium channel, containing the mutation sites, was amplified using two rounds of PCR in a thermocycler with a heated lid. Two rounds of PCR with nested primers provides an improved yield of a fragment for subsequent sequencing analysis. The primary round was carried out in 50μl using primers Aph1 (tggcccacactgaatctttt) and Aph12 (tcgatgagttgtgattcatgg) under the following conditions; 1μl genomic DNA, 1 μl of 10mM dNTP's, 2μl of each primer (100ng/ml), 5μl of 10x reaction buffer and 0.5 μl of Dynazyme™ II DNA polymerase (finnzymes). Thermal conditions were as follows; 94°C for 3 min, then 35 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 45 sec and finally an extension at 72°C for 10 minutes. The second round used primers Aph1 and Aph 16 (gttgtaggttctggatag). Iμl of the primary reaction was carried into the second reaction which was carried out under the same conditions as the first except that only 30 cycles of amplification were used. Sequencing was performed using the ABI Prism® BigDye™ terminator cycle sequencing ready reaction kit according to the supplier's standard procedure using primers Aph 15 (cggtggaacttcaccgatt) and Aph 21 (atactatcataaacgagtgc). The samples were analysed using an Applied Biosystems 310 automated DNA sequencer. Sequence data was aligned and analysed using Vector NTI (Informax Inc.).

Carboxylesterase and MACE testing was done according to published immunological and biochemical methods (Devonshire *et al.*, 1986; Moores *et al.*, 1994). The MACE mechanism is co-dominant so that both heterozygous and homozygous resistant insects show the same phenotype. The MACE assay is therefore unable to distinguish these individuals.

2.3 Results

Diagnostic Dose Assay

The diagnostic dose assay provides a cheap and effective means of screening populations of *M. persicae* for knockdown resistance. A range of deltamethrin concentrations were tested and 100ppm was found to give optimal discrimination between the genotypes conferring highest resistance. For the 100ppm diagnostic dose bioassay (Figure 2.1) a two-sided binomial test using the normal approximation method was used to test for differences in survival between genotypes. A comparison between those samples heterozygous for L1014F (kdr) and homozygous for M918 (SRSS), and the samples homozygous for L1014 and M918 (SSSS) indicated survivorship was not significantly different ($p=0.083$). Aphids that were heterozygous for L1014F and M918T (super-kdr) (SRSR) showed significantly higher survivorship at this dose of deltamethrin than those homozygous for F1014 alone (RRSS) ($p<0.001$) and those heterozygous for L1014F alone (SRSS) and those homozygous for L1014 and M918 (SSSS) ($p<0.001$). RRSS aphids showed, in turn, significantly higher survivorship than those heterozygous for L1014F alone (SRSS) and those homozygous for L1014 and M918 (SSSS) ($p<0.001$). A bioassay using 100ppm of deltamethrin would therefore able to distinguished the two genotypic classes (RRSS and SRSR) then available with the highest resistance to pyrethroids, but would not be able to distinguish between genotypes conferring lower or no resistance (survivorship proportions falling within the 95% confidence intervals of a genotype would be assigned to that genotype).

This bioassay was also applied to a clone collected from the field in 2003 that was found to be homozygous for both F1014 and T918 (RRRR). Over five replicates it gave an average of 98% survival, grouping with SRSR aphids. A higher dose of deltamethrin could possibly distinguish between genotypes heterozygous for M918T and homozygous for T918 but this has not been investigated.

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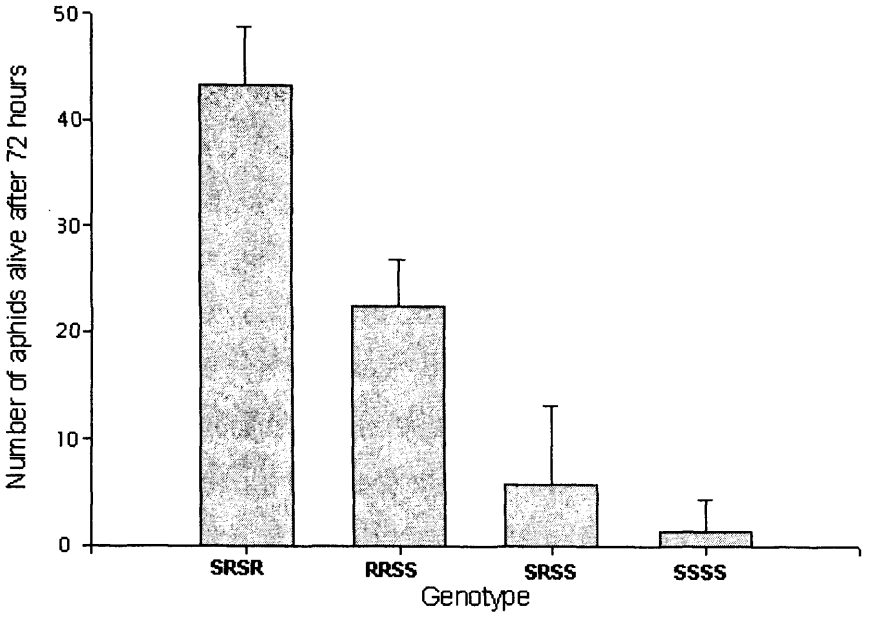


Figure 2.1 Mean survivorship of different *M. persicae* kdr and super-kdr genotypes to 100ppm deltamethrin for 25 field collected clones. SRSR: heterozygous M918T and L1014F, RRSS: homozygous for M918 and F1014, SRSS: homozygous for M918 and heterozygous for L1014F, SSSS: homozygous for both M918 and L1014. Error bars indicate 95% confidence intervals.

PCR Based Allelic Discrimination Assay Using Fluorescent Probes

Allelic discrimination assays using fluorescent dye-labelled probes are designed to run in a 96 well format with integrated software that automatically analyses the fluorescence data so that no post amplification manipulations are required. During amplification each probe binds specifically to its complementary PCR product. DNA polymerase then cleaves the reporter dye from the attached probe, which results in increased fluorescence of the reporter dye as it is separated from the quencher (figure 2.2). This cleavage occurs every cycle resulting in an increase of fluorescence proportional to the amount of PCR product. Two separate assays were run on the gDNA extracted from individual aphids; one using primers and probes that are selective for L1014 or F1014 (kdr), the other using probes and primers selective for M918 or T918 (super-kdr). The F1014 and T918 "resistant" probes were labelled with VIC reporter dye, whilst L1014 and M918 were labelled with FAM reporter dye. In either assay a substantial increase in VIC fluorescence alone indicates a homozygote for F1014 or T918, a substantial increase in FAM fluorescence alone indicates a homozygote for L1014 or M918 and a substantial increase in both signals indicates a heterozygote (figure 2.2). To determine the full genotype the end point fluorescence values for the two dyes are automatically corrected for background levels and plotted against each other in bi-directional scatter-plots (Figure 2.3). The allelic discrimination assay was verified as reliable by incorporating selected "control" aphids of known genotype (from DNA sequencing) on each plate of random field samples (Figures 2.3). The clustering of samples allowed for easy and accurate genotype scoring. Table 2.1 shows an example of screening data collected using this method in conjunction with MACE and carboxylesterase testing.

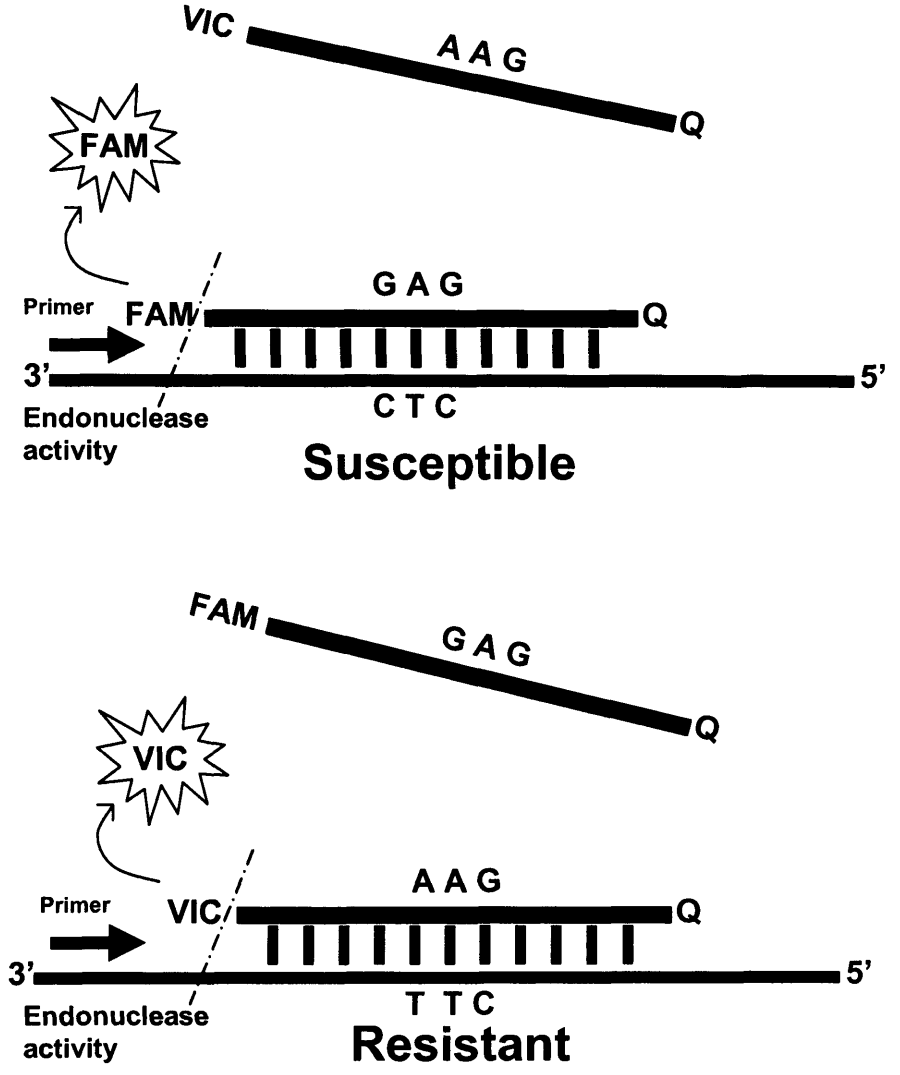


Figure 2.2. The principle of allelic discrimination using dye labelled probes. Probes were designed for susceptible and resistant alleles, each carrying a different dye (FAM for susceptible, VIC for resistant) and a quencher. During PCR amplification the probes bind only to their exact-match, allele fragments are then broken down by the 5' nuclease of the *Taq* polymerase. This releases the reporter dye from its quencher and results in an increase in fluorescence that enables the genotype to be scored.

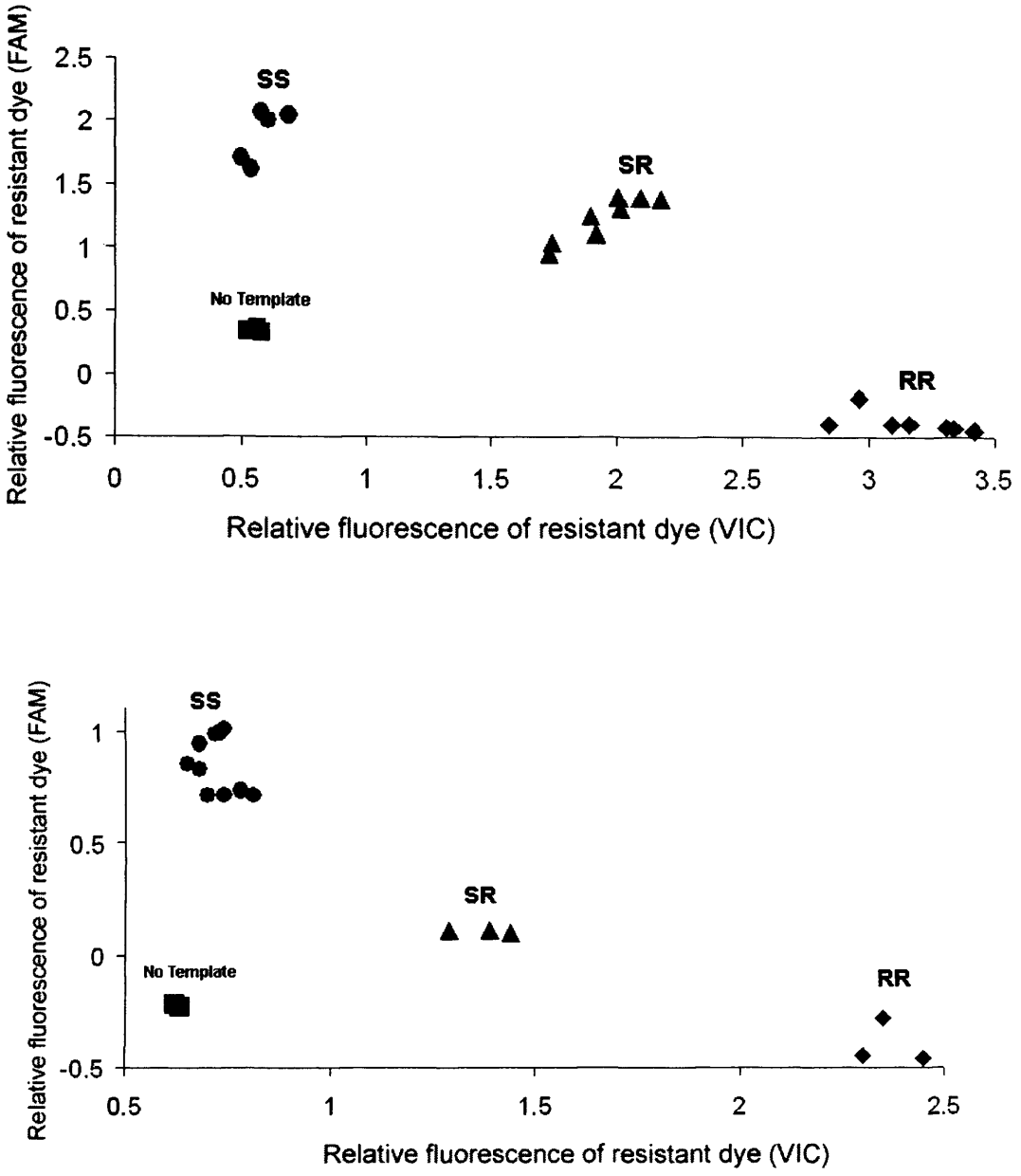


Figure 2.3. Scatterplots of final corrected fluorescence values in *M. persicae* using fluorescent probes specific for susceptible and resistant mutations

a. L1014F (kdr). SS: homozygous L1014, SR: heterozygous L1014F, RR: homozygous F1014.

b. M918T (super-kdr). SS: homozygous M918, SR: heterozygous M918T, RR: homozygous T918.

Table 2.1. Resistance genotypes of *M. persicae* samples collected from Scotland in 2001. Aphids are classified according to their kdr/super-kdr genotype, MACE genotype and carboxylesterase level.

kdr genotype	Super-kdr genotype	MACE	Esterase level			
			S	R1	R2	R3
SS	SS	No	40	6	2	0
SS	SS	Yes	0	0	0	0
SR	SS	No	2	3	1	0
SR	SS	Yes	0	3	0	2
RR	SS	No	1	0	0	0
RR	SS	Yes	0	0	0	0
SR	SR	No	0	2	3	5
SR	SR	Yes	0	0	1	25

2.4. Discussion

The ability to quickly and reliably determine the resistance status of a pest is an important tool in pest management. It allows for a rational choice of insecticide to be made based on the type and extent of resistance present. This would prevent the spraying of ineffective insecticides with the financial and environmental costs that entails, and also allows for the post-mortem of control failures. Knowing why control failures occurred may prevent a recurrence of that failure.

Although the diagnostic dose bioassay provides a cheap, low-tech means for assessing pyrethroid resistance phenotypes in *Myzus persicae*, it is difficult to establish a single dose that discriminates all of the possible genotypes. It is also slow (runs over 72 hours), requires live adult aphids in good condition and therefore requires the rearing of clonal lineages. PCR-based allelic discrimination techniques on the other hand enable accurate genotyping and

clear discrimination of heterozygotes and homozygotes. They can be performed on aphids of any life stage, including eggs, and even dead or preserved individuals. They require only a small fraction of a single aphid homogenate allowing tests to be run in conjunction with other assays. Allelic discrimination using quantitative real-time PCR has been used successfully in a number of systems (Monk *et al.*, 2002; McGuigan and Ralston, 2002; Sevall, 2001; Glaab and Skopek, 1999; Abbaszadegan *et al.*, 1997). The methods previously used for determining kdr and super-kdr genotypes in *M. persicae*: PCR amplification of specific alleles (PASA) (Guillemaud *et al.*, 2003a), single-strand conformation polymorphism (SSCP) and sequencing, are not high throughput and require post-amplification manipulations which increase the time and labour required. Single-strand conformation polymorphism (SSCP) has been tried with limited success for kdr but this technique does not yield consistent results on field collected aphids (unpublished results). The only potential drawback of PCR based allelic discrimination assay is that it can only be used to detect resistance alleles of known sequence. If another mutation were to arise that also conferred resistance, it would have to be characterised at the gene sequence level so that a third allelic discrimination assay could be developed alongside the existing assays.

The other major attraction of this technique is the ability to determine the presence or absence, in a single aphid, of all three resistance mechanisms of practical importance in *M. persicae*. Using this suite of assays it is possible to assign them to one of a possible 72 different resistance classes. This level of resolution is, to my knowledge, unprecedented for any resistant pest species to date. Table 2.1 shows data collected from Scottish populations of *M. persicae* where control failures had been reported. Many individual aphids in these populations were shown to possess kdr, super-kdr, elevated carboxylesterases and the MACE mutation, making them virtually immune to pyrethroids, carbamates and organophosphates used to control them. This is also the first time MACE, elevated carboxylesterase, kdr and super-kdr have all been recorded in an individual aphid. The occurrence of multiple resistance mechanisms in individuals is an increasing phenomenon with important implications for control strategies.

At present the carboxylesterase, kdr and MACE tests use 2%, 10% and 88% of the single aphid homogenate respectively, and the MACE test can be run successfully on 50% of the homogenate. This means that if other resistance mechanisms (or mutations) should arise and are characterised at the biochemical or molecular levels, there is potential for developing other diagnostics and including these alongside the existing assays.

Chapter 3.
Temporal and spatial incidence of insecticide resistance in *Myzus persicae*

Abstract

The aphid *Myzus persicae* is an important arable pest species throughout the world. Extensive use of insecticides has led to the selection of resistance to most chemical classes including organochlorines, organophosphates, carbamates and pyrethroids. Resistance to pyrethroids (knockdown resistance) is often the result of mutations in the *para*-type sodium channel protein. In *M. persicae,* knockdown resistance is associated with two amino-acid substitutions L1014F (kdr) and M918T (super-kdr). In this study the temporal and spatial distributions of these mutations, diagnosed using an allelic discriminating PCR assay, were investigated alongside other resistance mechanisms (MACE and elevated carboxylesterases). Samples were collected from the UK, Europe and Australia. The kdr mutation and elevated carboxylesterases were widely distributed and recorded from nearly every country. MACE and super-kdr were widespread in Europe but absent in Australian samples. The detection of a strongly significant heterozygote excess for both kdr and super-kdr throughout implies selection against individuals that are homozygous for these resistance mutations. The pattern of distribution found in the UK seemed to indicate strong selection against the super-kdr (but not the kdr) mutation in any genotype, in the absence of insecticide pressure.

It was also shown that there was significant association between the different resistance mechanisms which was probably promoted by asexual reproduction.

3.1 Introduction

The control of *M. persicae* is achieved primarily by the application of insecticides, often with multiple applications each year. This has led to the evolution of insecticide resistance conferred by three genetically-independent mechanisms (Devonshire *et al.*, 1998). The first of these to be characterised was the over-production of one of two closely related carboxylesterases (E4 and FE4). Depending on the amount of carboxylesterase produced, individuals are classified into one of four somewhat arbitrary categories: S (susceptible), R_1 (moderately resistant) R_2 (highly resistant) or R_3 (extremely resistant) (Devonshire *et al.*, 1986). This mechanism confers strong resistance to organophosphates and lower resistance to carbamates and pyrethroids.

Two types of target-site insensitivity conferring insecticide resistance have also been found in *M. persicae*. Individuals with modified acetylcholinesterase (MACE) show high levels of resistance to dimethyl carbamates such as pirimicarb and triazamate (Moores *et al.*, 1994). The MACE phenotype has recently been shown to be associated with a single amino acid substitution (serine to phenylalanine, S431F) within the active site of the enzyme (Nabeshima *et al.*, 2003; Andrews *et al.*, 2004). Two mutations in a voltage-gated sodium channel protein (kdr and super-kdr) conferring knockdown resistance to pyrethroids and DDT have also been identified in *M. persicae* (Martinez-Torres *et al.*, 1999b; Eleftherianos *et al.*, 2002). The two mutations are L1014F (kdr) and M918T (super-kdr), based on the housefly para sequences (Embl acc: X96668).

Resistance to pyrethroids in *Myzus persicae* has been recorded throughout Europe (Field *et al.*, 1997; Mazzoni and Cravedi, 2002; Guillemaud *et al.*, 2003b; Nauen and Elbert, 2003; Foster *et al.*, 1998; Field and Foster, 2002; Anstead *et al.*, 2004; Fenton *et al.*, 2005), and in the USA, Japan and Chile (Field *et al.*, 1997; Fuentes-Contreras *et al.*, 2004). Due to the low number of samples in most of these studies there are little meaningful data available on the frequency of resistance. Australian populations have been found to contain the 1,3 autosomal translocation associated with elevated E4 esterase (Wilson *et al.*, 2002), indicating this resistance mechanism is probably present. Prior to this study only a single clone (2169G), which was

collected in October 1997 from brussel sprouts in Lincolnshire, had been found with the M918T mutation (in conjunction with L1014F) (Eleftherianos *et al.*, 2002).

Collections from suction traps and field sites were utilised in this study. The use of suction traps reduces the sampling bias introduced by using a limited number of field samples. Insecticide use and the presence of a single host in monoculture are likely to have reduced the diversity within a particular field. Research has shown that populations caught in suction traps are representative of a large area (Taylor, 1979). Observations of *M. persicae* abundance from individual traps were found to be correlated over distances up to approximately 700km (Cocu *et al.*, 2005) indicating the population sampled should be representative of the population as a whole over these distances.

There is an underlying assumption made that insecticide resistance mutations do not restrict dispersal. Whilst this has not been tested directly, studies utilising both suction trap and field collected data indicate a broad similarity between the amount of resistance in field collected and suction trap collected samples (see UK 2003 results in table 3.3 in this chapter and Waters *et al.* 2002).

This chapter details the spatial and temporal patterns of kdr mutations in Europe and Australia and examine the relationship between kdr mutations and other resistance mechanisms.

3.2 Materials and Methods

3.2.1 Rothamsted Cultures

A number of live *M. persicae* clonal lines collected between 1974 and the present day are maintained in culture at Rothamsted Research. These clonal lineages were initially tested for knockdown resistance using a diagnostic dose bioassay with deltamethrin (chapter 2 and Anstead *et al.*,

2004). Presence of the super-kdr mutation was then confirmed by DNA sequencing.

3.2.2 Field Collections

Field samples were obtained from a variety of sources in Europe, Africa and Australia. Aphids from Europe and Africa were either shipped alive on plant material or in 95% ethanol. The ethanol preserved specimens could only be tested for kdr mutations as the MACE and carboxylesterase tests need active enzymes. Samples from Australia were shipped frozen in "solution 21" (25% glycerol, 0.5%Triton X-100, 100 mM KCl, 20 mM Tris (hydroxymethyl) aminomethane, 1 mM oxytetracycline, 10μM $CuSO_4$) (Tatchell *et al.*, 1988) preserving enzyme activity and DNA. Specimens were also obtained from Rothamsted's UK-wide network of suction traps (Woiwood and Harrington, 1994) and these were always shipped in alcohol. The Rothamsted suction trap is shown in figure 3.1. The four UK traps used were Preston (53° 51'16", 2° 45'47"), Long Ashton (51° 25'35", 2° 40'2"), Starcross (50° 37'44", 3° 27'13") and Wye (51° 11'50", 0° 56'21"). The trap locations are shown in figure 3.2. The Long Ashton trap was closed at the end of 2002 so collections were made from Preston in 2003. Aphids were collected from the three UK suction traps daily; up to two aphids a day were tested for kdr and super-kdr from each of the traps. A further twelve individuals were obtained from a suction trap in Sweden based on the design of the Rothamsted traps. European sample site locations are shown in figure 3.3. All the Australian samples were collected from the state of Victoria and these field locations are shown in figure 3.4. Field caught aphids from Europe were obtained from a number of co-operating organizations. These samples tended to be biased towards areas with treatment problems as there were more aphids present in these fields. To ensure the most representative sample from each field sample sheets were designed in conjunction with Syngenta and sent to farmers and Syngenta field staff. Figure 3.5 shows the sample sheets used.

Figure 3.1. A Rothamsted Insect Survey 12.2 m suction trap.

Figure 3.2. The locations of 12.2M suction traps used for the collection of *M. persicae* from the United Kingdom in 2002/3

Figure 3.3 The location of European *Myzus persicae* collection field sites and the Swedish suction trap 2001-2003. The number of individuals collected is indicated. Large circles indicate >10 individuals collected.

Figure 3.4 The location of *Myzus persicae* collected from the state of Victoria, Australia 2002 and the number of clonal lineages tested.

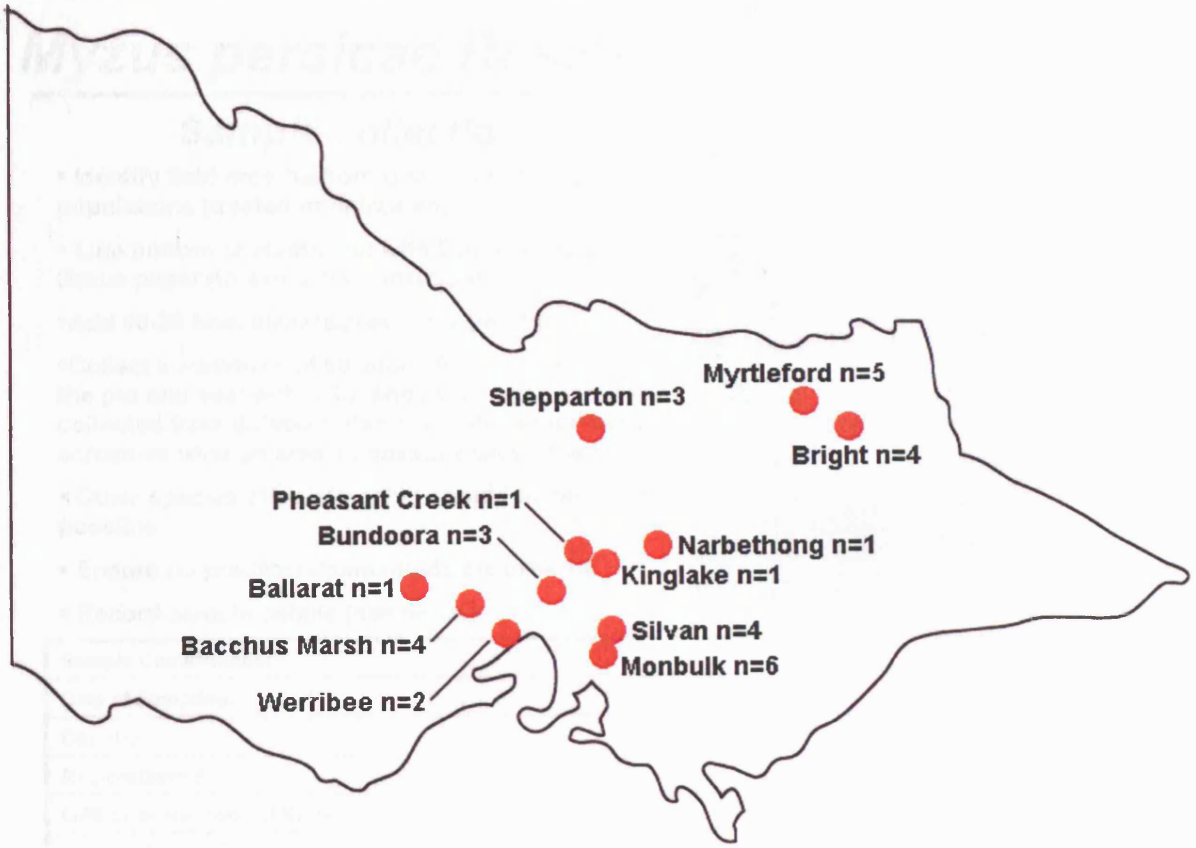


Figure 3.5 Sample sheet distributed to people interested in providing *M. persicae* samples.

Myzus persicae Resistance Monitoring

Sample collection

- Identify field crop harboring Myzus persicae populations (treated or untreated). Fig1.
- Line bottom of plastic pot with 2 to 3 layers of tissue paper (to avoid condensation).
- Add 10-20 host plant leaves in the pot. Fig 2.
- Collect a minimum of 50 adult *Myzus persicae* into the pot and seal with a lid. *Myzus persicae* should be collected from different plants distributed uniformly across as wide an area as possible within the field.
- Other species of potato aphid should be removed if possible.
- Ensure no predators/parasitoids are present.
- Record sample details (see below).

Fig1.

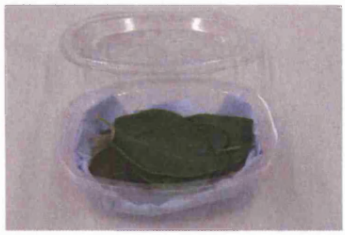


Fig2.

Sample Code/Number:	
Date of Sampling:	
Country:	
Region/District:	
GPS or postal code (If Known):	
Nearest Town:	
Collectors Name:	
Crop:	
Typical number of insecticide sprays for region:	
Previous insecticide treatment:	

- Place a copy of the sample details and the aphids into a padded envelope and post by special delivery or courier to the following address:

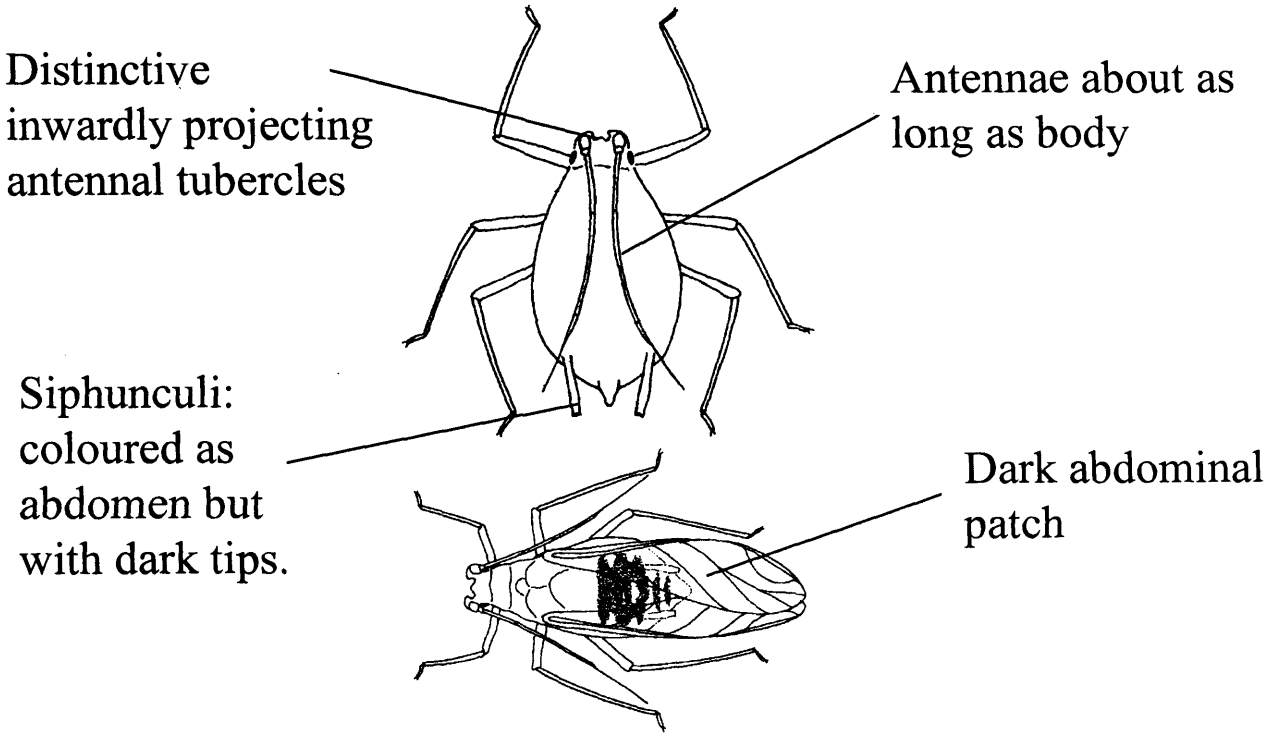
- **A copy of the Rothamsted DEFRA licence must accompany the shipment**

Copies of the DEFRA licence or general inquires can be directed to Russell Slater by e-mail ( ) or by telephone ( )

Figure 3.5

Continued

Myzus persicae



3.2.3 Testing for insecticide resistance

During 2001 testing for kdr and super-kdr mutations was performed using DNA sequencing or diagnostic dose bioassay (chapter 2 and Anstead *et al.*, 2004). From 2002 onwards the kdr and super-kdr mutations were diagnosed using a more rapid allelic discrimination assay (chapter 2 and Anstead *et al.*, 2004). Aphids were assigned to their genotypes according to the following codes SRSR: heterozygous at both sites, RRSS: homozygous resistant at kdr, susceptible at super-kdr, SRSS: heterozygous at kdr, susceptible at super-kdr, SSSS: homozygous susceptible at both sites, RRRR: homozygous resistant at both sites. MACE and carboxylesterase tests were performed using standard techniques (Devonshire *et al.*, 1986; Moores *et al.*, 1994). For genotypic testing single aphids were homogenised in 50µl of PBS/Tween (Phosphate buffer, 0.02M, pH 7.0 containing 0.05 v/v Tween 20) in the wells of a microtitre plate using a multihomogenizer (Ffrenchconstant and Devonshire, 1987). 1µl was used for carboxylesterase testing, 5µl was removed for the extraction of genomic DNA for diagnosing kdr and super-kdr and the remainder was used to test for the MACE phenotype.

3.2.4 Statistical analysis

Deviations from Hardy-Weinberg equilibrium at the kdr and super-kdr mutation sites were tested for in field collected samples where more than 10 aphids were available. Associations between elevated carboxylesterase, kdr, super-kdr and MACE were analysed using standard t-tests and analysis of variance (unbalanced design) (ANOVA). Linkage dis-equilibrium between the two kdr mutations and MACE was calculated using DIS (Dasmahapatra *et al.*, 2002), as were deviations from Hardy-Weinberg equilibrium. Data was pooled by country and year to increase the sample size for linkage disequilibrium calculations. The French and Australian samples were omitted from the linkage disequilibrium analysis as neither MACE nor super-kdr were found in these samples.

Table 3.2. Discriminating dose bioassay scores for clonal lineages maintained in culture at Rothamsted Research and tested with 100ppm deltamethrin. Survival indicates the proportion of 100 aphids surviving. Genotypes shown were demonstrated by sequencing

Clone	Date collected	Location	Survival	Genotype
1054B	17/7/1992	Japan	0.00	SRSS
3882B	2/12/1999	Staffordshire	0.00	
946E	1/10/1991	Norfolk	0.00	SRSS
1260G	28/9/1994	Greece	0.03	
3058A	29/10/1988	Cambridgeshire	0.01	SSSS
FrenchR	1/11/1979	France	0.06	SSSS
3174B	20/11/1998	Hereford	0.08	
3340A	9/6/1999	Sussex	0.09	
4255A	29/11/2000	Worcestershire	0.11	
2518A	1/6/1998	Turkey	0.13	
1267B	22/3/1995	Chile	0.14	
800F	1/6/1978	Italy	0.17	SSSS
2146K	11/7/1997	Greece	0.2	
2156A	22/7/1997	Cambridgeshire	0.21	SRSS
3172B	20/11/1998	Cambridgeshire	0.21	
3437B	13/7/1999	Shropshire	0.22	
4082A	21/7/2000	Shropshire	0.24	
1316A	1/10/1997	Hertfordshire	0.28	SRSS
2042H	24/10/1996	Lincolnshire	0.29	SRSS
1200Q	15/12/1993	Argentina	0.29	SRSS
3172A	20/11/1998	Cambridgeshire	0.32	SRSS
948D	1/6/1991	Bedfordshire	0.36	SRSS
3104B	5/11/1998	Cambridgeshire	0.38	RRSS
2043B	24/10/1996	Lincolnshire	0.4	RRSS
2042E	24/10/1996	Lincolnshire	0.41	RRSS
926B	13/7/1990	Greece	0.42	
794J	25/10/1994	England	0.44	RRSS
2144F	11/7/1997	Greece	0.51	
TIV	10/6/1975	Bedfordshire	0.48	RRSS
3495B	27/7/1999	Yorkshire	0.48	
4241B	3/10/2002	Yorkshire	0.48	
2012A	1/8/1996	Lincolnshire	0.77	SRSR
4268A	8/11/2000	Cambridgeshire	0.81	SRSR
4268B	8/11/2000	Cambridgeshire	0.81	SRSR
3110B	11/11/1998	Cambridgeshire	0.91	SRSR
3979A	10/7/2000	Essex	0.92	SRSR
2161C	10/9/1997	Lancashire	0.95	SRSR
2169G	1/10/1997	Lincolnshire	0.96	SRSR
2163E	17/9/1997	Derbyshire	0.97	SRSR

3.3 Results

3.3.1 Rothamsted cultures

Forty clones maintained at Rothamsted historical collection were tested in a diagnostic dose bioassay and nine had a survivorship of over seventy percent, indicating the likely presence of the super-kdr mutation (table 3.2). All were subsequently tested by sequencing and confirmed as heterozygous for the kdr and super-kdr mutations. All nine clones were collected in England between 1996 and 2000.

3.3.2 Geographical distribution of resistance

A summary of the distribution of resistance mechanisms is shown in table 3.3. Elevated carboxylesterase was the most widespread resistance mechanism and was present in all samples. The kdr mutation was also widely found, only being absent in the Zimbabwean sample. MACE and the super-kdr mutation were both absent in samples from Australia and Zimbabwe. There were considerable differences between the frequencies of resistance mechanisms in the samples, probably due to different insecticide selection pressures. Kdr was present at frequencies between 0.19 and 0.66 in all but two of the samples; it was absent in aphids from Zimbabwe and was present, as a homozygote, in all the aphids from Sweden. Super-kdr was present at much lower frequencies, and was absent from a number of samples including some that were relatively large (e.g. samples collected from Germany in 2002 and 2003). MACE was present in most samples, but was not found in the Australian samples. In some samples, especially those from Greece and Italy, selection seems to have completely removed the lower carboxylesterase categories and all aphids collected scored as R2 or R3.

Table 3.3. Geographical incidence of various resistance mechanisms. Carboxylesterase levels are divided into the 4 standard categories of resistance. Kdr and super-kdr are shown as allele frequencies. MACE is shown as the frequency of the MACE phenotype, (i.e. homozygotes and heterozygotes combined).

Country	n	Resistance Frequency						
		Knockdown resistance		Carboxylesterase				
		kdr	super-kdr	S	R1	R2	R3	MACE
2001								
UK	96	0.26	0.19	0.45	0.15	0.07	0.33	0.32
France	8	0.66	0.66	0.17	0.17	0.33	0.33	0.13
2002								
Australia	36	0.36	0	0.64	0.28	0.08	0	0
France	23	0.44	0	0.22	0.78	0	0	0
Germany	83	0.19	0	0.10	0.63	0.25	0.04	0.05
Greece	71	0.41	0.22	0	0	0.34	0.66	0.84
Italy	15	0.5	0.3	0	0	0.67	0.33	0.53
Scotland	12	0.42	0.05	0	0.5	0.47	0.03	0.38
UK trap[a]	57	0.42	0.06					
Sweden Trap[a]	12	1	0					
2003								
Zimbabwe[a]	15	0	0					
France[a]	85	0.42	0.33					
Germany	14	0.43	0	0	0.36	0.57	0.07	0
UK trap[a]	194	0.28	0.06					
UK	146	0.39	0.03	0.04	0.45	0.44	0.06	0.39

[a]Samples collected and subsequently stored in alcohol were not tested for carboxylesterase levels or for MACE

3.3.3 UK suction trap catches in 2002 and 2003

Figures 3.6 and 3.7 show the total number of *M. persicae* caught by the three suction traps used in 2002 and 2003. The bi-modal distribution is typical of *M. persicae* in the UK where two major flights per season occur (Karley *et al.*, 2004). This pattern can be seen in the data from each trap (figures 3.8 and 3.9), although the timing and magnitude of the flights varied between traps and years. In 2002, the highest total number of aphids was collected from Starcross (92), then Wye (74) and Long Ashton (32). In 2003 higher total numbers were collected from Wye (318) than Preston (131) and Starcross (54). Overall aphid numbers were higher in 2003 and this was reflected at each trap.

Figures 3.10 and 3.11 shown the incidence of knockdown resistance detected in the combined trap catches for 2002. Figure 3.10 shows the genotypic frequencies and figure 3.11 shows the frequency of each resistance mutation. Figures 3.12 and 3.13 show the equivalent data for 2003. Figure 3.14 shows the genotypic frequency for each trap individually for 2003 only. In both 2002 and 2003 the kdr mutation was present during every month, although the frequency varied considerably; ranging from 5% (August 2003) to 65% (October 2002). In 2003 it was found during every month at every trap, with the exception of two months at the Preston trap when only a single aphid was recorded (figure 3.14). It was found most commonly as a heterozygote without the super-kdr mutation (SRSS). At the beginning of the 2003 season it was present at a higher frequency than at the end of 2002 indicating it persists between seasons. In contrast the super-kdr mutation was only detected during August and September 2002 and June and September 2003. In June 2003 when the super-kdr mutation was at a frequency of 0.15, aphids with this mutation were being caught at all three traps (figure 3.14). However in September, super-kdr aphids were only caught at Preston. The two most common genotypes were SSSS (no resistance mutations) and SRSS (heterozygous for kdr, homozygous susceptible for the super-kdr mutation) (table 3.4). In all the samples (field and suction trap) no individual was found carrying the super-kdr mutation alone. It was always accompanied by the kdr mutation.

Table 3.4. Frequencies of knockdown resistance genotypes of *M. persicae* caught in UK suction traps during 2002 and 2003

Genotype	2002 (n=53)	2003 (n=154)
SSSS	28%	49%
SRSS	47%	36%
RRSS	13%	3%
SRSR	11%	9%
RRRR	0%	2%

Figure 3.6. Combined total number of *M. persicae* collected from three UK suction traps (Starcross, Long Ashton and Wye) in 2002.

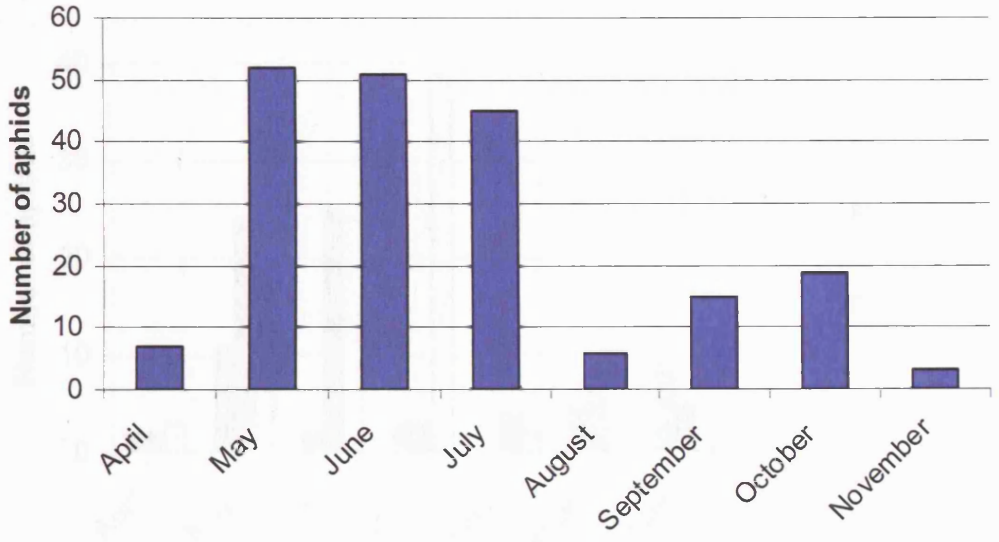


Figure 3.7. Combined total number of *M. persicae* collected from three UK suction traps in (Starcross, Long Ashton and Preston) 2003

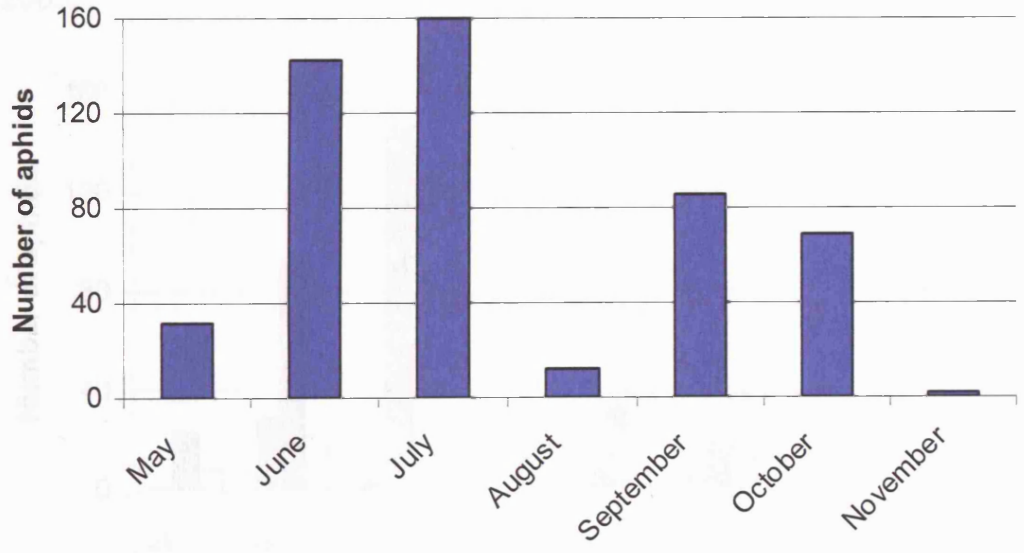


Figure 3.8. Total number of *M. persicae* collected from three UK suction traps in 2002.

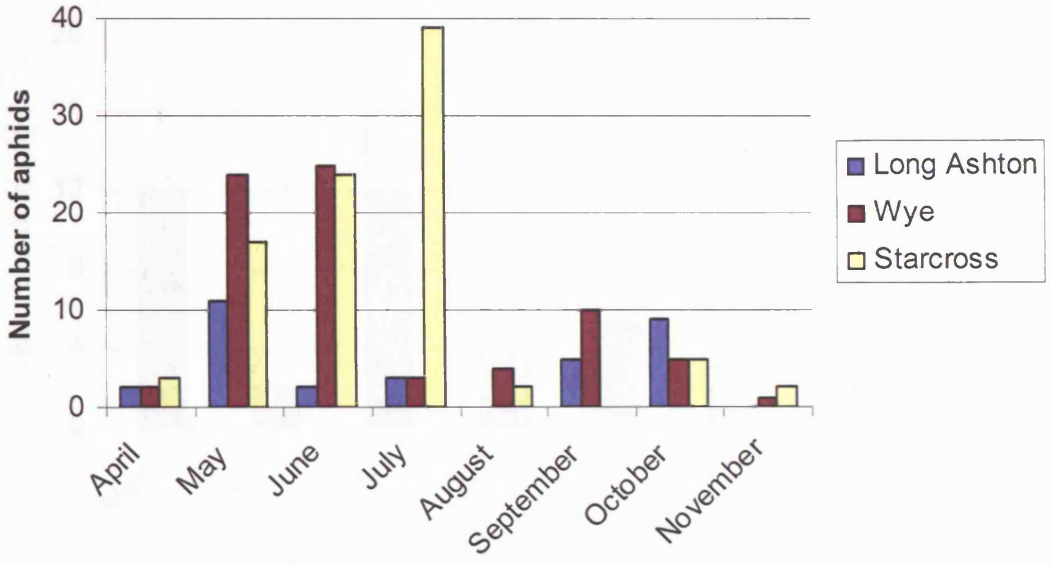


Figure 3.9. Total number of *M. persicae* collected from three UK suction traps in 2003.

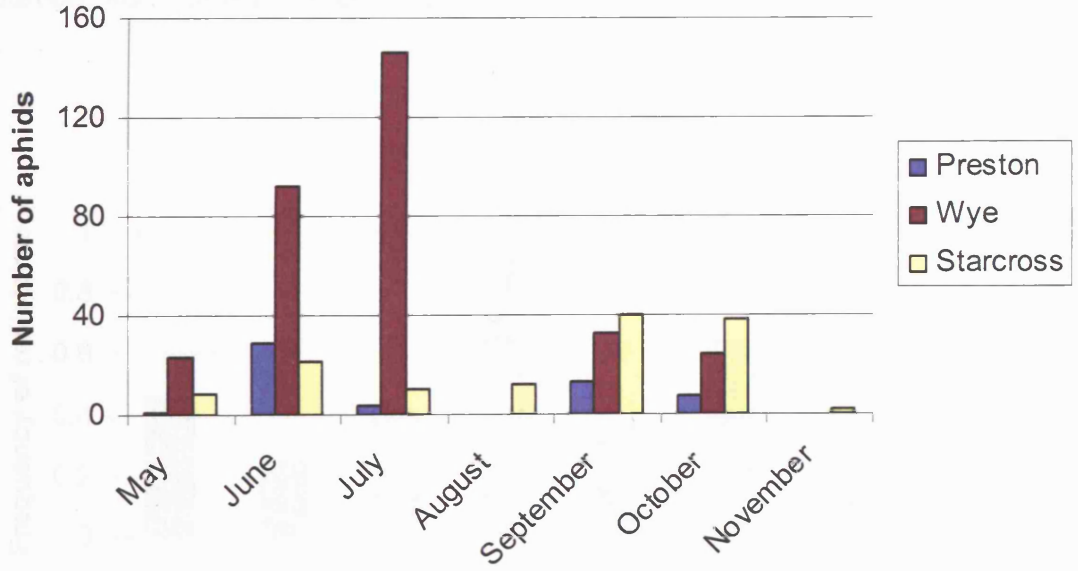


Figure 3.10. Knockdown resistance genotypes of *M. persicae* collected from three UK suction traps in 2002. SRSR: heterozygous at both sites, RRSS: homozygous resistant at kdr, susceptible at super-kdr, SRSS: heterozygous at kdr, susceptible at super-kdr, SSSS: homozygous susceptible at both sites

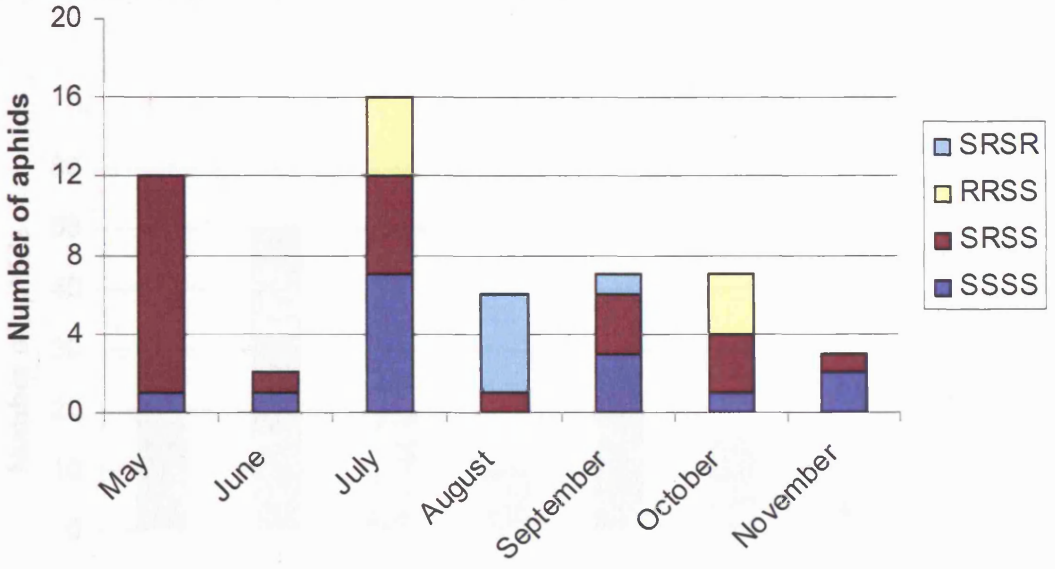


Figure 3.11. Knockdown resistance mutation frequencies in *M. persicae* collected from three UK suction traps in 2002. S-kdr: Super-kdr resistance mutation, kdr: kdr resistance mutation

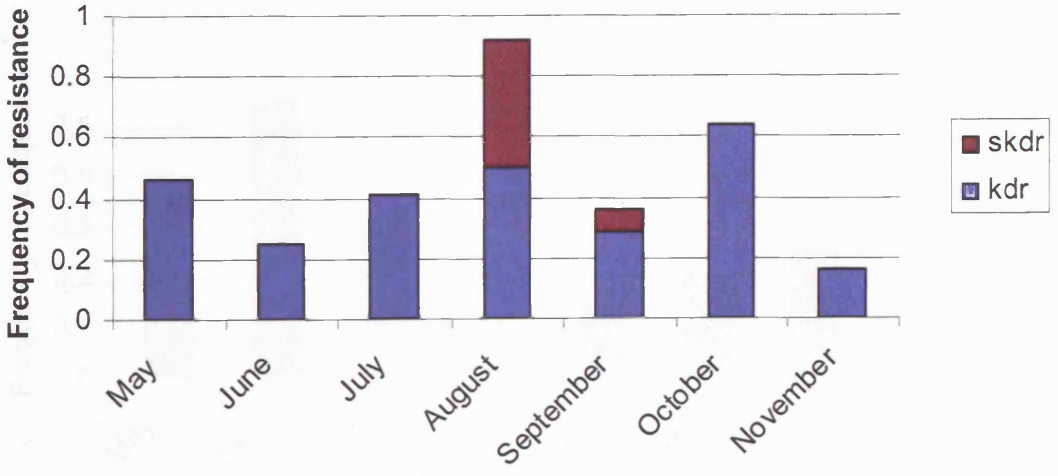


Figure 3.12. Knockdown resistance genotypes of *M. persicae* collected from three UK suction traps in 2003. SRSR: heterozygous at both sites, RRSS: homozygous resistant at kdr, susceptible at super-kdr, SRSS: heterozygous at kdr, susceptible at super-kdr, SSSS: homozygous susceptible at both sites, RRRR: homozygous resistant at both sites.

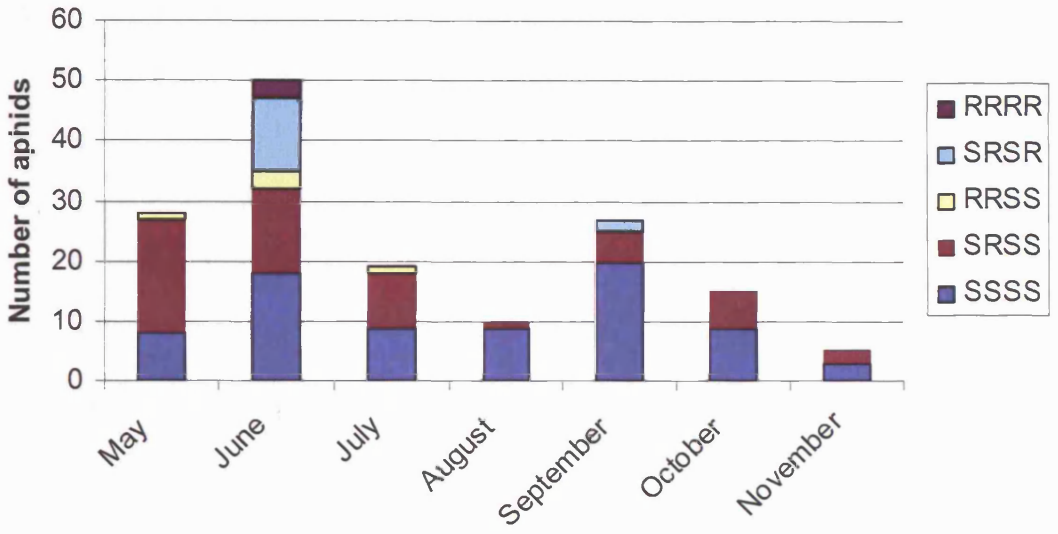


Figure 3.13. Knockdown resistance mutation frequencies in *M. persicae* collected from three UK suction traps in 2003. S-kdr: Super-kdr resistance mutation, kdr: kdr resistance mutation

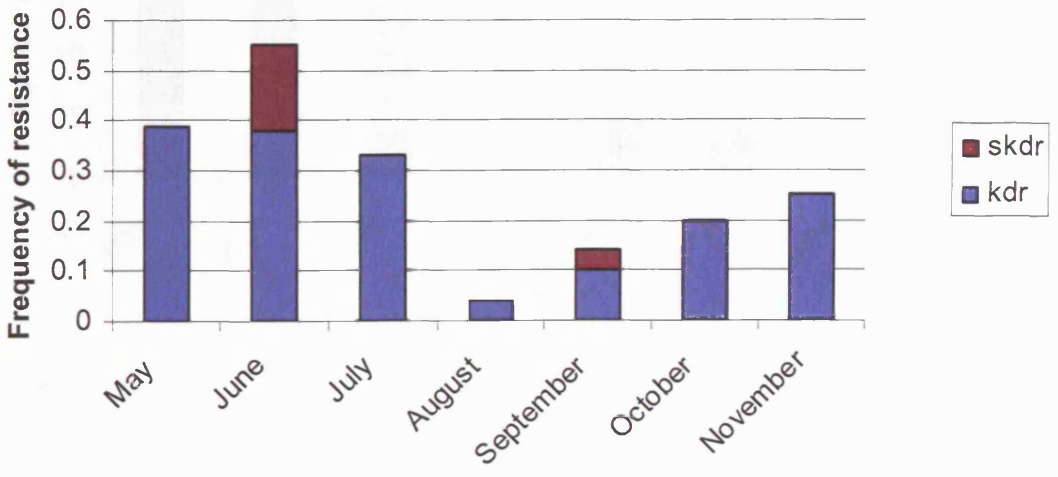
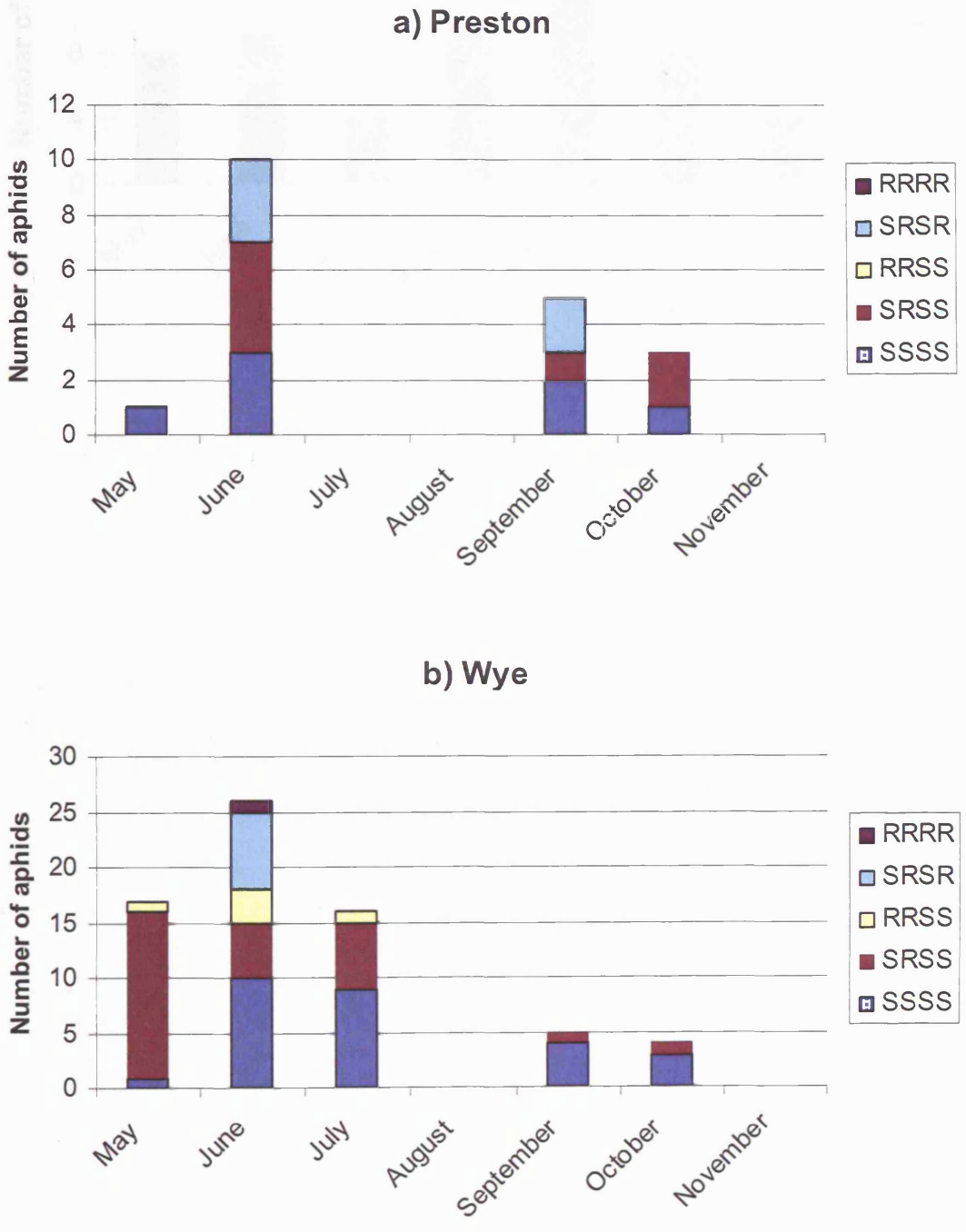


Figure 3.14. Knockdown resistance genotypes of *M. persicae* collected from the three UK suction traps in 2003. SRSR: heterozygous at both sites, RRSS: homozygous resistant at kdr, susceptible at super-kdr, SRSS: heterozygous at kdr, susceptible at super-kdr, SSSS: homozygous susceptible at both sites, RRRR: homozygous resistant at both sites.



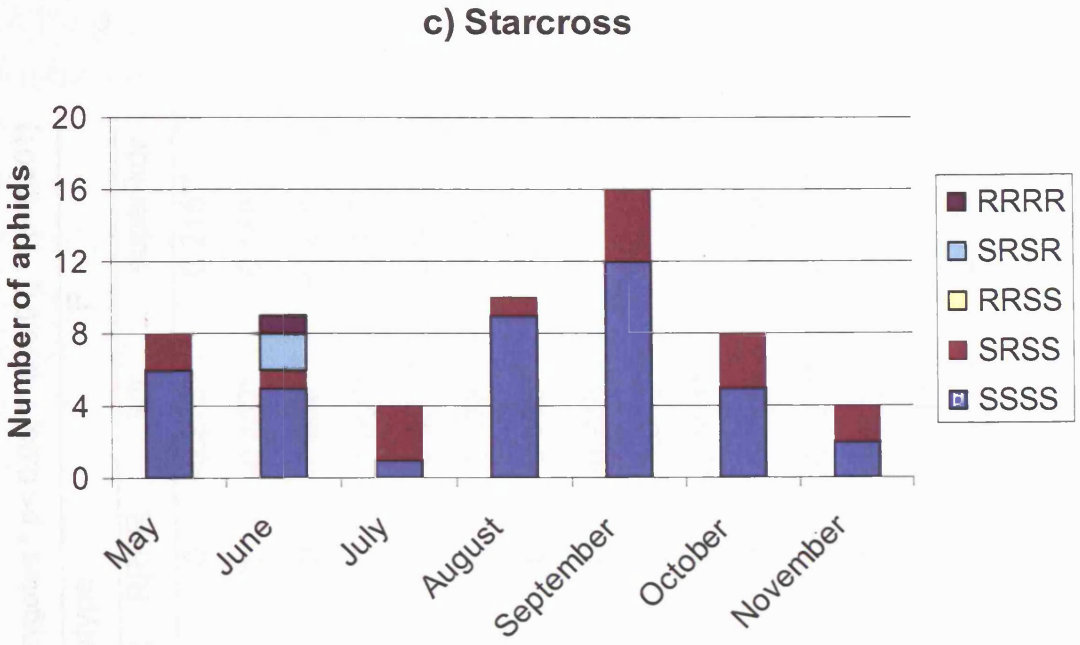

c) Starcross
Number of aphids
20
16
12
8
4
0
May
June
July
August
September
October
November
RRRR
SRSR
RRSS
SRSS
SSSS

Table 3.5. Tests for Hardy-Weinberg equilibrium of knockdown resistance mutations in field samples of *M. persicae*. The frequency of each resistant genotype is also shown. (A negative value for F represents a deficiency of homozygotes * p< 0.05, ** p<0.01, ***p<0.001)

Country	Year	Location	Crop	n	Knockdown resistance genotype					F	
					SSSS	SRSS	RRSS	SRSR	RRRR	kdr	super-kdr
UK	2001	Dundee	Mixed	99	0.52	0.12	0.01	0.35	0	-0.275**	-0.215**
Greece	2002	Karditsa	Tobacco	19	0.37	0.32	0	0.32	0	-0.462*	-0.188
Greece	2002	Meliki	Tobacco	20	0.2	0.35	0	0.45	0	-0.637**	-0.286
Italy	2002	Caserta	Peach	15	0	0.4	0	0.6	0	-1.000***	-0.429*
Greece	2002	Lehonia	Peach	20	0.1	0.2	0.1	0.6	0	-0.653**	-0.546**
Greece	2002	Meliki	Peach	19	0.11	0.37	0.11	0.42	0	-0.478*	-0.226
France	2002	Allonnes	Potato	13	0.08	0.92	0	0	0	-0.868***	-
Germany	2002	Ruhlsdorf	Potato	27	0.67	0.33	0	0	0	-0.238	-
Scotland	2002	Arbroath	Brassica	12	0	1	0	0	0	-1.000***	-
Germany	2002	Ahlum	OSR	47	0.64	0.36	0	0	0	-0.221*	-
UK traps	2002		Trap	53	0.26	0.48	0.13	0.12	0	-0.280*	-0.059
France	2003	Nimes	Peach	115	0.16	0.16	0.03	0.64	0.01	-0.621***	-0.454***
England	2003	Wakefield	Sprouts	50	0.04	0.82	0	0.14	0	-0.923***	-0.075
England	2003	Wilmington	Cabbage	24	0.60	0.40	0	0	0	-0.243	-
UK traps	2003		Trap	154	0.49	0.37	0.03	0.09	0.01	-0.196**	0.123

3.3.4 Hardy-Weinberg equilibrium

Deviations from Hardy-Weinberg equilibrium were calculated from samples collected from single fields and from the UK trap samples in 2002 and 2003 (pooled for the whole year) (table 3.5). The samples varied from Scottish samples where, it is believed, no sexual recombination occurs to Greek peach samples, which were spring-collected, where the aphids had just gone through a cycle of sexual reproduction. In all bar one instance these calculations showed a heterozygous excess, although the level of statistical significance varied.

3.3.5 Relationship between mechanisms

Figures 3.15 and 3.16 show the relationship between carboxylesterase level and other resistance mutations. To improve statistical power, rather than assigning individuals to the usual four categories of carboxylesterase level (S,R1,R2,R3), the continuously distributed carboxylesterase immunoassay values were used (Devonshire *et al.*, 1986). Figure 3.15 shows the mean carboxylesterase levels for aphids collected in 2002 and 2003 with and without the MACE mutation (error bars indicate 95% confidence limit). Aphids containing the MACE mutation had mean carboxylesterase levels significantly higher (equivalent to R2) than those without it (equivalent to R1) ($t=9.7$, $p<0.001$).

Figure 3.16 shows the mean carboxylesterase levels for *M. persicae* collected in 2002 and 2003 with different kdr genotypes. According to an analysis of variance ($p<0.05$, 3 df, LSD=0.4, $F<0.001$) SSSS, SRSS and RRSS aphids had significantly lower carboxylesterase levels than SRSR. SSSS and SRSS aphids had significantly lower carboxylesterase levels than RRSS aphids. There was no significant difference in carboxylesterase levels between SSSS and SRSS aphids. SRSR aphids had a mean carboxylesterase activity equivalent to R3, RRSS to R2 and SSSS/SRSS to R1.

An analysis of linkage disequilibrium was done for samples for which data were available on kdr, super-kdr and MACE (table 3.6). The kdr and super-kdr mutations were very strongly linked. There was significant linkage disequilibrium between MACE and kdr and MACE and super-kdr in the UK.

There was no significant linkage disequilibrium between MACE and kdr in Germany (super-kdr was absent) and in Greece and Italy there was no significant linkage disequilibrium between MACE and kdr or super-kdr.

Figure 3.15. The relationship between carboxylesterase level and the presence of the MACE mutation in *M. persicae*, error bars indicate 95% confidence of the mean (mace n=129, non-mace n=161).

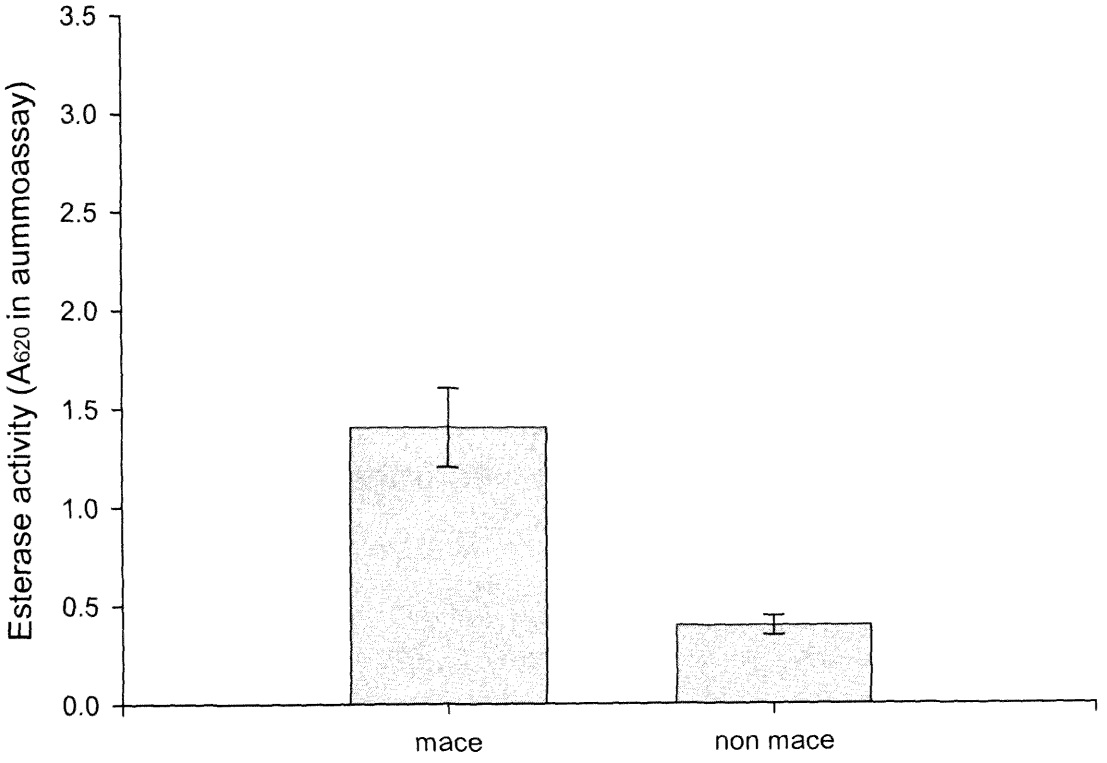


Figure 3.16. The relationship between kdr genotype and carboxylesterase level in *M. persicae*, error bars indicate 95% confidence of the mean (SSSS n=86, SRSS n=148, RRSS n=8, SRSR n=44).

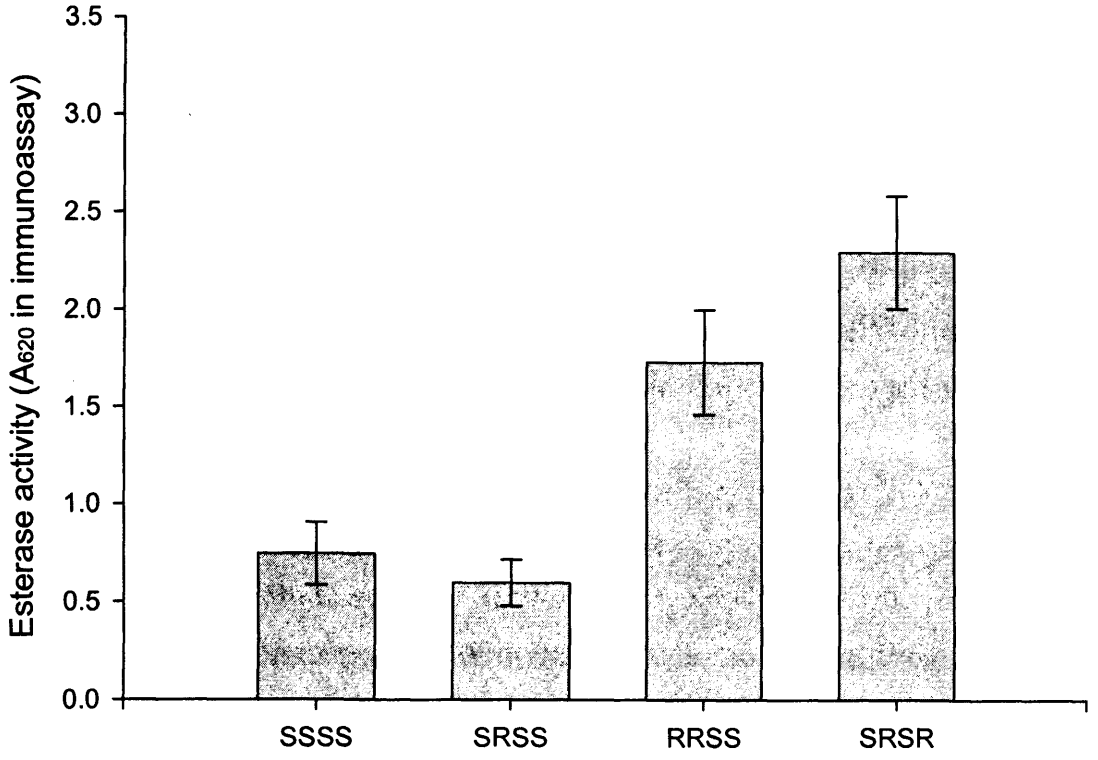


Table 3.6. Values for correlation coefficient for linkage equilibrium (* p< 0.05, ** p<0.01, ***p<0.001)

Country	n	Kdr/super-kdr	Kdr/MACE	Super-kdr/MACE
		R	R	R
UK 2001	99	+0.81***	+0.46***	+0.41***
UK 2003	146	+0.21*	+0.41**	+0.13*
Germany	83	-	+0.40	-
Greece	71	+0.64***	-0.10	-0.11
Italy	15	-	-	+0.39

3.4 Discussion

3.4.1 Laboratory cultures

The analysis of the laboratory cultures confirmed the presence of super-kdr in a number of UK samples and showed it to be present at least one year earlier than previously thought. The earliest clone with a super-kdr mutation was 2012A which was collected from potato in Lincolnshire in August 1996 (2169G which was the earliest previously recorded sample with super-kdr was collected from Lincolnshire in 1997 (Eleftherianos, 2003)). These results also confirmed the utility of the discriminating dose bioassay as a preliminary indicator of knockdown resistance genotype (Anstead *et al.*, 2004). Clones showing >70% survival were subsequently confirmed as possessing the super-kdr mutation.

3.4.2 Population dynamics of *M. persicae*

Host-alternating aphids such as *M. persicae* typically undergo two migratory events during a season, one in spring as aphids move from their primary to their secondary host and one in the autumn when they return (Taylor, 1979; Weisser, 2000). This pattern is seen in *M. persicae* caught in 2002 and 2003. However in both years a single trap (Starcross in 2002 and Wye in 2003) shows a peak in July which is later than expected for a spring migration event. This peak is likely to be due to the production of alates on secondary hosts. Aphid alates on secondary hosts are normally produced in response to over-crowding or reduced host-quality (for a review see Muller *et al.* 2001).

3.4.3 Spatial and temporal spread of resistance

The kdr mutation (L1014F) was well distributed both temporally and spatially. It was present in all the countries sampled with the exception of Zimbabwe. The L1014F mutation has now been found in *M. persicae* from every continent where this species is known to occur. Elevated carboxylesterases were also common in all the countries sampled, in some cases to the exclusion of susceptible aphids. MACE and super-kdr were both found throughout Europe but were not found in samples from Australia and Zimbabwe. The Zimbabwean sample was a small sample from a single field,

so no conclusions can be drawn about the possible absence of kdr, super-kdr or MACE from this country. The samples from Australia were collected from a number of different sites and hosts and represented the most common clones found in the state of Victoria (Cristoph Vorburger personal comm.). Some of these samples had also been subject to intense selection with pyrethroids and carbamates. If the super-kdr mutation and/or MACE were present in Victoria it would have been present in these samples.

There was a strong contrast in the temporal patterns between the kdr and super-kdr mutations in the UK suction trap catches. The kdr mutation was very common throughout the season; being present every month and often in 30% of individuals or more. The super-kdr mutation appeared in samples from August and September 2002 and from June and September 2003. Numbers were too low to disclose clear trends in the occurrence of this mutation but a plausible explanation is that numbers build up under selection by pyrethroids but then decline in the absence of insecticide pressure. This would imply that super-kdr in *M. persicae* carries a fitness cost in the absence of insecticide selection. The kdr mutation has been shown to be associated with deleterious fitness effects in *M. persicae*. Foster *et al.* (2003b) showed kdr and elevated carboxylesterases were associated with a reduced response to alarm pheromone. *M. persicae* with elevated levels of carboxylesterase also have added fitness costs at low temperature in the laboratory which might be expected to decrease the over-wintering ability of *M. persicae* individuals in the field (Foster *et al.*, 2003c). These data shows no evidence of such a cost for kdr individuals as there was a higher proportion of kdr resistant individuals in the spring of 2003 (39%) than the previous autumn (18%).

Overall there was less resistance in 2003 than in 2002 (table 3.4). This was most likely due to the dynamic between selection with insecticides and fitness costs. It is likely more pyrethroids were used in 2002 which lead to a higher proportion of resistance, as spraying decreased in 2003 the non-resistant clones (SSSS) were favoured leading to a proportional increase in their numbers. Such annual swings are likely to be a feature of *M. persicae* populations as insecticide pressures change between years.

3.4.4 Hardy-Weinberg equilibrium

In all the fields sampled both kdr and super-kdr were in heterozygous excess, and in most cases this excess was a significant deviation from Hardy-Weinberg expectations (see table 3.5). This heterozygous excess indicates there is selection against homozygous individuals for both kdr and super-kdr. Homozygous susceptible *M. persicae* are most likely selected against by the application of insecticides as kdr gives some resistance to pyrethroids when present as a heterozygote in *M. persicae* (Foster *et al.*, 2002a), and individuals which are heterozygous at both mutations are unaffected by high doses of pyrethroids (Eleftherianos *et al.*, 2002). Homozygous resistant individuals are very rare for both kdr and super-kdr. This may be because of fitness costs associated with resistance, either as a direct result of the resistance or because deleterious recessive mutations are associated with the resistance mutation. There are known fitness costs associated with the kdr mutation in *M. persicae.* The presence of kdr is associated with a decreased response to alarm pheromone, which could render them more vulnerable to predation or parasitism (Foster *et al.*, 2003c). It should be noted that in these experiments homozygous resistant and heterozygous resistant aphids showed a similar reduced response to alarm pheromone. If homozygous resistant individuals are selected against a much higher potential cost associated with the mutation for these individuals compared to heterozygotes would be expected. Homozygous excess in microsatellites has been the general rule in *M. persicae* populations studied to date (Fenton *et al.*, 2003; Wilson *et al.*, 2002; Guillemaud *et al.*, 2003c). Homozygous excess has also been found in French *Sitobion avenae* populations (Simon *et al.*, 1999) although heterozygous excess has been found in *Rhopalosiphum padi* (Delmotte *et al.*, 2002) and various *Sitobion* species (Wilson *et al.*, 1999; Sunnucks *et al.*, 1996). As these loci are selectively neutral, selection could not have caused this deviation. Delmotte *et al.* (2002) proposed two alternate hypotheses to explain the heterozygous excess they found: allele sequence divergence during long-term asexuality or the hybrid origin of asexual lineages.

3.4.5 Missing genotypes

During the sampling only some of the possible genotypic combinations of the two mutations conferring kdr resistance were detected. The super-kdr mutation was only ever found in conjunction with kdr. The most likely explanations for this are either that super-kdr arose in one or more alleles already containing the kdr mutation, and little or no recombination has occurred between the mutations, or that the presence of the super-kdr mutation alone is highly dis-advantageous (see Morin *et al.* 2002). In either case only three alleles would be expected to predominate; fully susceptible, resistant at kdr but susceptible at super-kdr, and resistant at both sites. These three alleles form six possible genotypes of which only five were found. No individuals were found which were homozygous resistant for kdr and heterozygous for super-kdr (RRSR). If Hardy-Weinberg equilibrium was assumed across all the populations sampled in table 3.5 approximately 5% of the individuals (or n=34) should be RRSR. One problem with determining if the absence of this genotype is significant is that there isn't any information available on the clonal diversity within these samples. However, the relationship between the number of individuals and the number of clonal lineages has been explored in other studies. If these samples follow the same pattern as those in Fenton *et al.* 1998 for instance there would be approximately 200 separate clonal lineages. The chance of failing to detect one RRSR individual from 200 randomly selected clonal lineages if they were present at 5% of the population is <0.005. In fact the proportion of RRSR individuals is likely less than 2% ($p<0.01$).

This genotype has been created during laboratory crossing experiments (Eleftherianos, 2003), so its absence from these field samples is surprising. There are a number of possible explanations. It's possible that these populations are considerably less diverse than expected and there are a low number of clonal lineages being sampled repeatedly. If this were the case the resolution of the study may be too low to pick up a rare genotype (although RRRR individuals were collected which should theoretically be rarer). The other possible explanations are that there is assortative mating, which can lead to a deficit of particular genotypes (extremely unlikely in my view, and anyway mating is a very rare event) or selection is reducing the number of

individuals with this genotype and the resolution of this study was too low to detect them. Given that both kdr and super-kdr are likely to have fitness costs associated with them (see chapter 1 and 5), this seems the most likely explanation for the absence of this genotype.

3.4.6 Links between mechanisms

As expected there was strong linkage dis-equilibrium shown between kdr and super-kdr. These are two mutations of the same gene and as such are strongly physically linked. It is also not surprising to see an association between the levels of carboxylesterase and the kdr and super-kdr resistance mutations since both give resistance to pyrethroids. Elevated levels of carboxylesterase sequester and detoxify the pyrethroid and the kdr and super-kdr reduce the sensitivity of the voltage-gated sodium channel. Here both should be selected for by insecticide pressure and in asexual populations would be likely to stay associated. The association between esterase and MACE is less pronounced. MACE aphids have an average R2 esterase level and non-MACE have R1. The association between MACE and esterase and its state of linkage disequilibrium with the two sodium channel mutations is likely related to asexual reproduction as it is not physically linked to either. If a population of asexually reproducing clones are sprayed with a pyrethroid and then a carbamate, only those with both sets of resistance will survive leading to linkage dis-equilibrium within the population. Some popular insecticides are even mixtures of a carbamate and a pyrethroid e.g. Dovetail is a mixture of pirimicarb and lambda-cyhalothrin. This conclusion is confirmed by the fact that in Southern Europe, where *M. persicae* can reproduce sexually, the linkage between MACE and kdr/super-kdr breaks down (see table 3.6). It is interesting note that in this case a single annual recombination event is enough to remove any linkage dis-equilibrium.

Chapter 4.

Evidence for multiple origins of identical resistance mutations in the aphid *Myzus persicae*

Abstract

The peach-potato aphid Myzus persicae (Sulzer) (Hemiptera: Aphididae) has developed resistance to pyrethroid insecticides as a result of a mechanism conferring reduced nervous system sensitivity, termed knockdown resistance (kdr). This reduced sensitivity is caused by two mutations, L1014F (kdr) and M918T (super-kdr), in the para-type voltage gated sodium channel. Kdr mutations in *M. persicae* are found in field populations world-wide. In order to investigate whether this situation is due to the mutations arising independently in different populations or by single mutation events that have spread by migration, regions flanking these mutations were sequenced from different geographical areas. The DNA sequences produced, which included a 1kb intron, were found to be highly conserved. Several different haplotypes were identified containing kdr and super-kdr. Whilst these results could indicate either multiple independent origins of both mutations or recombination following a single origin, given the short timescale of resistance development, multiple independent origins of kdr and super-kdr is the most plausible interpretation.

4.1 Introduction

The emergence of insecticide resistance mechanisms is a good example of selection driven by human activities. The number of independent origins of insecticide resistance traits is an important question for those studying the evolution of resistance and its subsequent spread. Several studies have tested a single global origin hypothesis for mechanisms conferring insecticide resistance. Sequences from regions flanking the co-amplified A2 and B2 esterase genes in *Culex pipiens* were shown to be identical regardless of where the sample was collected, indicating that after an initial amplification event, the resistance gene had spread through migration (Raymond *et al.*, 1991; Guillemaud *et al.*, 1996). A single origin has also been reported for DDT resistance in *Drosophila melanogaster* (Daborn *et al.*, 2002). In this study resistance to DDT was always associated with over-transcription of a single Cyp6g allele, coding for a cytochrome P450 monooxygenase and characterized by the insertion of an Accord transposable element. All resistance alleles had an identical DNA sequence, despite the presence of numerous different susceptible alleles. In *Myzus persicae* the DNA sequences flanking amplified genes coding for the carboxylesterases E4 and FE4 were found to be identical in *M. persicae* and *M. nicotinae* indicating a single amplification event that had spread between the two species (now considered host-adapted races) (Field *et al.*, 1994).

The origin of resistance mechanisms that involve target-site mutations has also been investigated for example, cyclodiene resistance or Rdl (resistance to dieldrin) is conferred by single point mutations in a gene coding for a subunit of a GABA receptor, in this case the same resistance mutation in this gene of *Tribolium castaneum* was shown to have multiple origins which had then spread throughout the world (Andreev *et al.*, 1999). This study showed several haplotypes that differed only in the presence or absence of the resistant mutation. Multiple origins of resistance can also provide evidence of genetic isolation between isolated taxa. For instance, analysis of DNA sequence within the *Ace1* gene (coding for the insecticide target acetylcholinesterase) of *Culex pipiens sub a* and *C. pipiens sub b* indicated a separate origin for a mutation causing resistance in each subspecies (Weill *et*

al., 2003). Conversely an analysis of DNA sequence data flanking the *rdl* resistance mutation in *Bemisia tabaci* supported a unique origin for this mutation in different biotypes (Anthony *et al.*, 1995b).

Pyrethroid resistance in *M. persicae* is a world-wide phenomenon. Both the kdr and super-kdr mutations have been found in a number of widespread *M. persicae* populations (see chapter 3). The kdr mutation in particular was found in populations all across the UK, Europe and from Australia. It has also previously been reported from the USA and Japan (Field *et al.*, 1997). An important question for the study of this resistance mechanism and others is how this distribution arose. Do resistance causing mutations arise independently multiple times in separate populations? Or do mutations move between populations by migratory gene-flow?

The kdr mechanism is well suited for a study of the origins of insecticide resistance as it has been subject to selection since the 1940's when DDT was first introduced. In the late 1940's DDT was the chemical of choice for the control of both the peach tree borer (*Sanninoidea exitiosa* (Say)) and the oriental fruit moth (*Grapholita molesta* (Busck)) (Bobb, 1949; Chochran, 1949) on peach trees (the primary host of *M. persicae*). *M. persicae* was also likely to have been under selection pressure for the development of DDT resistance on its secondary hosts as DDT was used on crops such as beans, beets, cabbage, cantaloupe, cucumber, onion, peas, potato, squash, tomatoes and turnips (Carter, 1948) , all of which are secondary hosts for *M. persicae*.

To investigate the number of possible independent origins for kdr and super-kdr, sequence data were collected from an intron approximately 1kb long that starts 3 bp downstream of the kdr mutation and 350 bp downstream of the super-kdr mutation. The intron 2 sequence was chosen for sequencing as previous work looking at approximately 70 sequences had shown only one variable site within the coding sequence and a smaller intron located between the two mutations (figure 4.1 shows the relative locations of the introns).

4.2 Materials and Methods

Aphid Sampling

Aphids were obtained from a number of sites and were chosen to maximise the likely diversity of resistant genotypes based on known resistance and geographical location (table 4.1). Live samples were shipped in Perspex "Blackman" boxes (Blackman, 1971) on potato or Chinese cabbage leaves. Asexual lineages were started from a single adult from these samples and reared on Chinese cabbage (*Brassica napus* var *chinensis* cv "Wong-Bok) leaves in leaf boxes in controlled environment rooms at 20 ± 1°C under 16:8 hours (light: dark). *M. persicae* were also collected from 12.2 metre suction traps operated by the Rothamsted Insect Survey (Harrington and Woiwod, 1994). Samples caught in the traps were initially stored in 50% ethanol. They were then transferred to Rothamsted (normally within one working day) where they were placed in 70% alcohol until they had been identified to species level by the survey team. *M. persicae* from the samples were then placed in 100% ethanol at 4°C for longer-term storage.

PCR, cloning and sequencing

Single aphids were homogenised in 50µl of PBS/Tween (Phosphate buffer, 0.02M, pH 7.0 containing 0.05 v/v Tween 20) in the wells of a microtitre plate using a multihomogenizer (ffrench-Constant and Devonshire, 1987). Genomic DNA was extracted from the homogenized samples using Dynazol® (Helena Biosciences) at a fifth scale. Aphids were scored for kdr resistance mutations using allelic discriminating PCR (see chapter 2 and Anstead *et al.*, 2004).

A 1.1 Kb DNA fragment was amplified using two rounds of nested PCR. The first PCR was done in a 50µl volume using Aph1 (TGGCCCACACTGAATCTTTT) and Aph22 (TACGAGCCAAAATTACTCAG) under the following conditions; 1µl genomic DNA, 1 µL of 10mM DNTP's, 5µl of each primer (100ng/ml), 5µl of 10x reaction buffer and 0.5 µl of Dynazyme II DNA polymerase. Thermal conditions were as follows; 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 2 min 30 sec, followed by a final extension at 72°C for 10 minutes. The second round used primers Aph5 (TTACACGTCGGAGAACC) and Aph22. Iµl of the primary reaction was carried into

the second reaction which was done under the same conditions as the first except that only 30 cycles of amplification were used. These PCR products were then divided; one half was sequenced directly and the other was ligated into a Pgem-T easy vector (Promega) and transformed into XL1-Blue supercompetent cells (Stratagene).

Plasmids were purified using the Wizard Plus SV miniprep kit (Promega). Sequencing was performed in both directions using two plasmid primers, M13 forward and M13 reverse, and two primers specific for the *M. persicae* sodium channel, Aph 10 (CACGATCATACTTAATTGT) and Aph 11 (TAAACCTACAAGTTAGTC). The samples for direct sequencing were sequenced using Aph 10, Aph11 and Aph 5 and analysed using an Applied Biosystems 310 automated DNA sequencer. Sequence data was aligned and analysed using Vector NTI (Informax Inc.).

Phylogenetic analysis

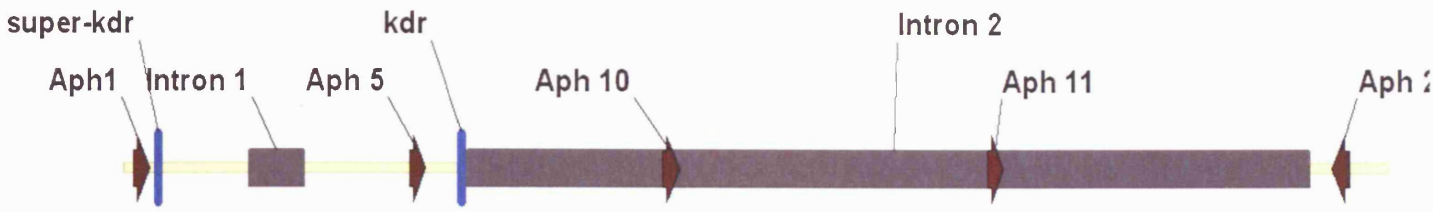
To determine the genealogical relationship between alleles sequence data they were analysed using the maximum likelihood method in Phyllip 3.6 (Felenstein, 2004). Global rearrangements were allowed and the input order was randomised (10x jumble), the transition/transversion ratio was 0.7. Bootstrap values were calculated using 100 tree iterations under the same conditions.

Table 4.1 Location and resistance genotypes of samples chosen for sequencing

Sample	Year collected	Country	Location	Esterase	MACE[1]	kdr	Super-kdr
162	2001	Scotland	Harlaw	R3	R	SR	SR
189	2001	Scotland	Gogar	R3	R	SR	SR
195	2001	Scotland	Waterside	R3	S	SR	SR
194	2001	Scotland	Kinnaird	S	S	SS	SS
21A	2001	Scotland	Dundee	S	S	SS	SS
S107.3	2003	England	Starcross	Unknown	Unknown	RR	SS
W4603C	2003	England	Wye	Unknown	Unknown	RR	RR
M66	2002	Australia	Shepparton	R1	S	RR	SS
11	2002	Australia	Ballarat	S	S	SR	SS
C7	2002	Australia	Werribee	R2	S	RR	SS
02IT8	2002	Italy	Caserta	R2	S	SR	SS
02 Melp19	2002	Greece	Meliki	R2	R	RR	SS
Meltob20	2002	Greece	Meliki	R3	R	SR	SR
Meltob8	2002	Greece	Meliki	R3	R	SR	SR
02 lehp12	2002	Greece	Lehonia	R2	R	SR	SR
A6	2002	France	Nimes	R3	Unknown	RR	RR

[1] Resolved to phenotype only since the kinetic assay does not distinguish reliably between SR and RR genotypes

Figure 4.1. Location of primers and introns within the 1.1kb region of the *M. persicae* sodium channel gene containing the kdr and super-kdr mutations.



4.3 Results

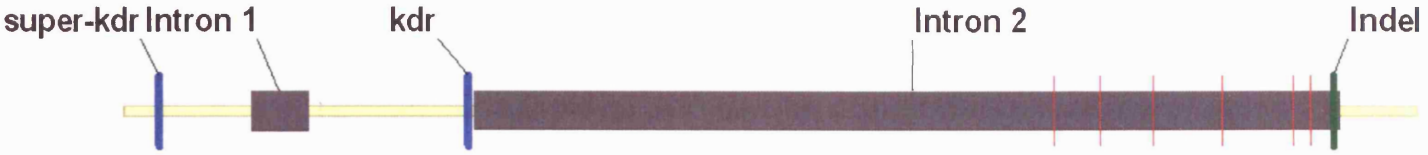
Sequencing data

The direct sequencing data were used to correct PCR errors caused by the occasional mis-incorporation of an oligonucleotide by Dynazyme. The mis-incorporation rate was approximately $5x10^{-4}$ errors per bp. As expected approximately three quarters of the samples had both complementary alleles cloned using three cloned fragments. The remainder had to be re-cloned several times. Only one allele was ever cloned for Mel20.

The intron DNA sequence was very conserved. There was a single four bp insertion/deletion (indel) which was found in two samples (IT8 and Meltob8) and there were six single nucleotide polymorphisms (SNP's). There were twelve haplotypes from the possible 128 which could be formed by six SNP's and one indel. Many samples shared haplotypes (table 4.2). There were five different haplotypes (C, E, F, K, L) containing the kdr mutation and three of these (E,F,K) also contained the super-kdr mutation. One of these kdr-containing haplotypes (L; from M66, S107.3 and 11akdr) differed from a susceptible haplotype (K) by only the kdr mutation. The haplotypes varied in frequency from haplotype C and I, which were both represented in about 20% of samples, to a number of haplotypes which were only found once. Figure 4.2 shows the location of the variable bases and indel. The variable bases were

all closest to the 3' end of the intron. Nucleotide variation across the sequenced region was 0.002 and substantially lower than expected. A similar study found 48 variable bases and indels within a shorter fragment (694bp) of DNA spanning the exon containing the rdl mutation and a flanking intron in *Tribolium castaneum* (Andreev *et al.*, 1999), corresponding to a nucleotide diversity of 0.01 – 0.006.

Figure 4.2. Location of variable bases within the 1.1kb region of the *M. persicae* sodium channel gene containing the kdr and super-kdr mutations.



Phylogenetic analysis

A single example of each haplotype was used for the phylogenetic analysis. There were two major clades shown (figure 4.3) and haplotype A did not group with the rest of the sequences. Both clades contained sequences with the kdr mutation alone and in combination with the super-kdr mutation. However the bootstrapping analysis indicated that these clades are not well supported, clade 1 being 46% supported and clade 2 being 63% supported. This tree structure is consistent with either multiple origins of resistance for kdr and super-kdr or substantial recombination within this sequence (or both). There was no evidence of geographical structuring within the tree; both clades contained haplotypes collected from Australia, Southern and Northern Europe.

Figure 4.3. Unrooted maximum likelihood tree of cloned *M. persicae* haplotype sequences, produced from nucleotide sequences from a 1.1kb portion of the para-type sodium channel gene. The percentages of 100 bootstrap replications supporting each branch are shown. *=contains the kdr mutation, **=contains the kdr and super-kdr mutation.

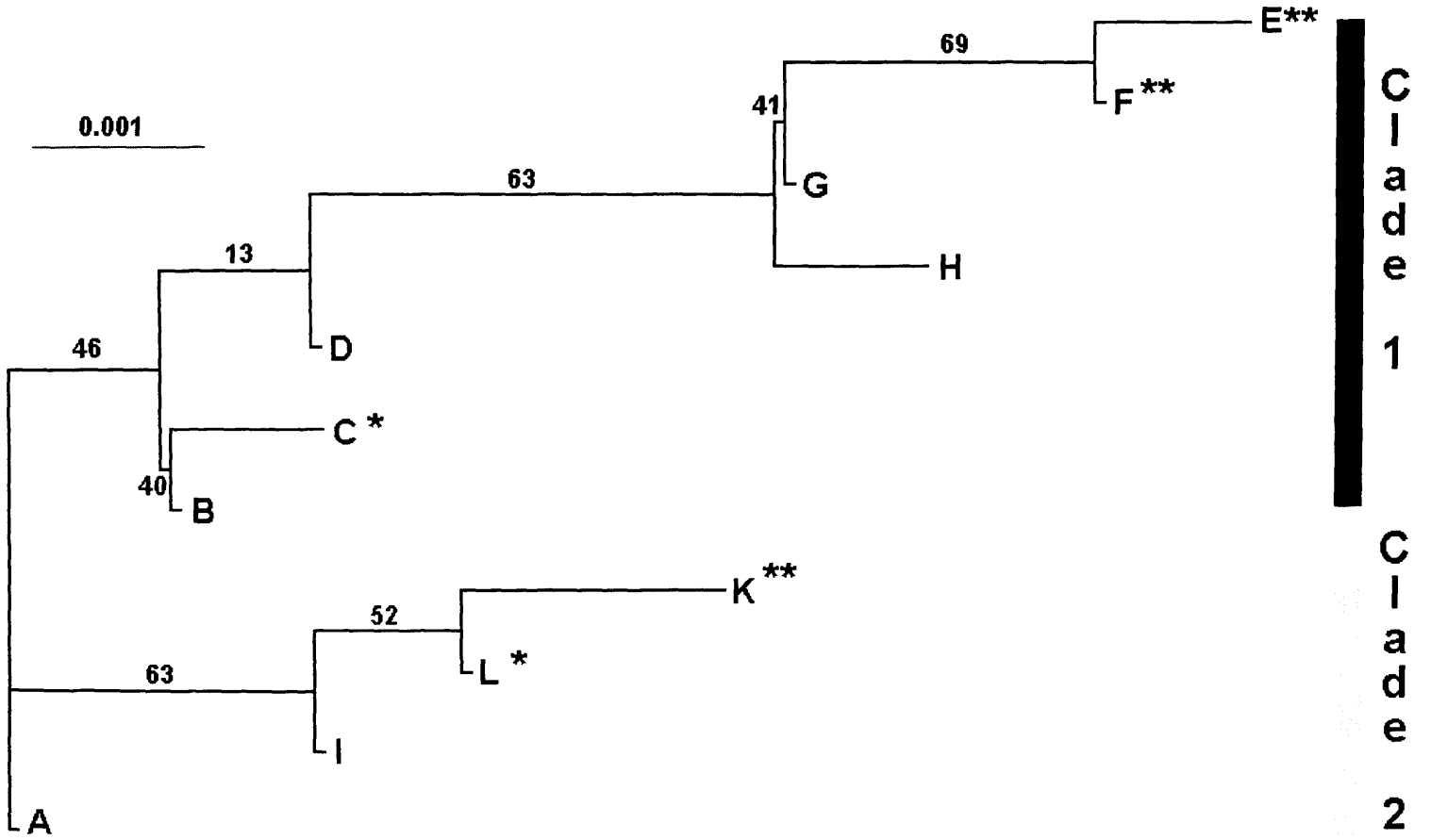


Table 4.2. Variable bases within an intron sequence adjacent to the super-kdr/kdr mutations in *M. persicae.* Kdr, T=resistant, super-kdr, C=resistant, X=4 base-pair insertion (AGTA).

Clone	Super-kdr	Kdr	724	777	838	917	999	1036	1046	Haplotype
162	A	C	C	T	A	T	G	T		A
189	A	C	C	T	A	T	T	T		B
195	A	C	C	T	A	T	T	T		B
Mel20	A	C	C	T	A	T	T	T		B
C7	A	T	C	T	A	T	T	T		C
C7	A	T	C	T	A	T	T	T		C
S107.3	A	T	C	T	A	T	T	T		C
It8	A	T	C	T	A	T	T	T		C
Melp19	A	T	C	T	A	T	T	T		C
Meltob8	A	C	A	T	A	T	T	T		D
189	C	T	A	G	T	A	G	T		E
Lehp12	C	T	A	G	T	A	T	T		F
194	A	C	A	G	T	A	T	T		G
194	A	C	A	G	T	A	T	T		G
M66	A	C	C	G	T	A	T	T		H
Lehp12	A	C	A	T	A	T	G	C		I
21	A	C	A	T	A	T	G	C		I
11	A	C	A	T	A	T	G	C		I
It8	A	C	A	T	A	T	G	C	X	J
Meltob8	A	C	A	T	A	T	G	C	X	J
162	C	T	A	T	A	T	G	C		K
195	C	T	A	T	A	T	G	C		K
A6	C	T	A	T	A	T	G	C		K
A6	C	T	A	T	A	T	G	C		K
W4603	C	T	A	T	A	T	G	C		K
W4603	C	T	A	T	A	T	G	C		K
M66	A	T	A	T	A	T	G	C		L
S107.3	A	T	A	T	A	T	G	C		L
11	A	T	A	T	A	T	G	C		L

4.4 Discussion

Since the introduction of DDT in the 1940's there has been prolonged selection pressure for the spread of resistance mutations of its target-site, the para-type voltage-gated sodium channel in many important pest species. Pyrethroids also target this receptor and have been used since the 1970's, when pyrethroids with high photo-stability (e.g. cypermethrin and deltamethrin) were developed (Leahey, 1979). Pyrethroid use then increased dramatically on field crops and pyrethroids today account for approximately 25% of the world insecticide market (Hemingway *et al.*, 2004). As a result mutations in the sodium channel conferring resistance to these insecticides have arisen in many insect pests representing all the major insect orders. Kdr is well documented in *M. persicae*. The first kdr mutation (L1014F) was reported in 1999 (Martinez-Torres *et al.*, 1999b), but the resistant clones used in this study were collected as early as 1990 and a clone in culture at Rothamsted (TIV collected in Bedfordshire) which is homozygous for L1014F was collected in 1975 (unpublished data). The super-kdr mutation (M918T) was initially discovered in a clone designated 2169G, collected in October 1997 from Lincolnshire in the UK (Eleftherianos *et al.*, 2002), however examination of clones collected earlier showed its presence in 1996 (clone 2012A also from Lincolnshire, see chapter 2). It therefore seems likely that the kdr mutation at least was present in field populations of *M. persicae* for some years before its detection.

The presence of a number of different haplotypes with resistance mutations could have come about in one of two ways. Either there have been a number of independent de-novo mutations arising in these haplotypes (e.g. haplotype B gave rise to haplotype C by a single mutation at the kdr locus) or recombination between a single progenitor for each mutation and other haplotypes gave rise to multiple resistant haplotypes e.g. a recombination event between B and K (resistant) would give rise to a new resistant haplotype (haplotype C) and a susceptible haplotype (H). Whilst it is possible to test for recombination within a set of sequences (Piganeau and Eyre-Walker, 2004), the amount of variation within this data-set was too low to be informative (Piganeau – pers, comm.). I therefore consider the likelihood of each possibility in turn.

The presence of several haplotypes that differ only in the presence/absence of the kdr mutation (e.g. biotype B/C and I/L) is consistent with multiple origins of this mutation. The occurrence of these "progenitors" has been used as

evidence of independent origins of rdl resistance in *T. castaneum* (Andreev *et al.*, 1999). The situation for super-kdr is not quite as clear-cut as there is only a single progenitor. Haplotype K differs from haplotype L only by the super-kdr mutation. However haplotypes E and F, the only others with the super-kdr mutation, appear related to each other but so different from K that they are likely to have had a separate origin. This difference is shown more clearly in figure 4.3; E and F are in clade 1 and K is in clade 2. It can be argued that the lifecycle of *M. persicae* also makes independent de-novo mutations the likely source of these resistant haplotypes rather than sexual recombination. *M. persicae* can undergo more than 20 asexual cycles a year and only a single sexual cycle (van Emden *et al.*, 1969). In areas (e.g. Northern Europe) where peach, the primary host, are largely absent there is believed to be no sexual cycle at all. Even where conditions are conducive to holocycly some populations may not produce sexuals. For instance some Greek populations feeding on tobacco are almost exclusively composed of three genotypes that are parthenogenic (Margaritopoulos *et al.*, 2003). This overall bias towards asexual reproduction argues in favour of independent de-novo mutations especially if these mutations are of relatively recent origin. The assumption that resistance arose recently is supported by the number of other species showing exactly the same pyrethroid-resistance polymorphisms. The L1014F mutation has been found in *Musca domestica* (Williamson *et al.*, 1993), *Blattela germanica* (Dong, 1997), *Haematobia irritans* (Guerrero *et al.*, 1997), *Plutella xylostella* (Schuler *et al.*, 1998), *Anopheles gambiae* (Martinez-Torres *et al.*, 1998), *Culex pipens* (Martinez-Torres *et al.*, 1999a) and *Anopheles sacharovi* (Luleyap *et al.*, 2002). M918T has been found in *M. domestica* (Williamson *et al.*, 1996) and *H. irritans* (Guerrero *et al.*, 1997).

The possibility of recombination giving rise to a number of resistant haplotypes from a single original cannot be excluded due to low variation in the intron sequence. In this scenario a single mutation a kdr containing haplotype which through intra-genic recombination formed a number of resistant haplotypes. The super-kdr mutation then arose in either the original or one of the recombined haplotypes. There does not seem to have been any recombination between the super-kdr and kdr mutations as extensive testing has failed to discover an allele with the super-kdr mutation alone. In this theoretical scenario it seems likely that haplotype K arose from haplotype L by mutation as it is

identical except for the presence of the super-kdr mutation. It is plausible that recombination could have formed all the resistant haplotypes found. A recombination event between B (susceptible) and L (resistant) would give rise to a new resistant haplotype (haplotype C) and a susceptible haplotype (H). Similarly a recombination event between K (resistant) and G (susceptible) would give rise to F (resistant) and I (susceptible) and a recombination event between haplotype A (susceptible) and F (resistant) would give rise to haplotypes E (resistant) and B (susceptible). The main argument however, against this conclusion is that there have probably been very few sexual generations for this amount of recombination. There have only been a maximum of 64 sexual generations since the first possible exposure to DDT (one per year between 1940 and 2004) and far fewer over the period that pyrethroids have been used intensively. In addition areas where *M. persicae* is wholly or largely anholocyclic experience no recombination at all. Thus the likelihood of recombination being the primary cause of multiple resistant haplotypes depends primarily on the absolute age of the mutations that now occur widely in contemporary populations of *M. persicae*. Whilst this is unknown the apparent chronology of resistance development is consistent with the theory these haplotypes arose recently by independent de-novo mutations. The kdr mutation was detected in the field 20 years before super-kdr and to date super-kdr has never been found independently of kdr. This indicates super-kdr has arisen in a kdr haplotype and there has been no recombination between these sites since as super-kdr would have been detected on its own if this had happened.

Several haplotypes had a very wide geographical distribution. Haplotype C was collected from Australia, England, Italy and Greece and haplotype I was found in Greece, Scotland and Australia. Given that the intron sequence should be selectively neutral this implies extensive migration and therefore gene-flow between populations. Haplotype C (containing the kdr mutation) has spread to many areas, probably facilitated by the global trade in plant material. In contrast the super-kdr haplotypes (K, E and F) seemed more geographically confined. Haplotype K was found 6 times, but only in England and Scotland, E and F were each found once, in Scotland and Greece respectively.

Chapter 5.
Concluding discussion

5.1 Introduction

Ever since the first widespread use of synthetic insecticides in the 1940's there has been a constant evolutionary pressure on insects to develop resistance to the chemicals used to kill them. Resistance development in *M. persicae* is a microcosm of this process with resistance to a number of insecticide classes including pyrethroids now well documented and characterised. By 1997, 63 species had developed resistance to this class of insecticides (Tomlin 1997) and the present number probably stands at over 100.

Insecticide resistance has huge costs. It results in lost crop yield, wasted sprays and it means new pesticide chemistries have to be developed entailing huge expense. The development of resistance is particularly troubling in the case of the pyrethroids as their relatively low cost, high efficacy and low mammalian toxicity make them well suited to use in a wide range of settings including developing countries where the cost of pesticides is a major constraint in the expansion of agriculture.

5.2 Resistance management

There are two main aims of resistance management, or as it might better be termed mitigation (Hoy, 1998): to delay the appearance of resistance and to control it after its appearance to increase the lifetime and efficacy of control agents. The most effective way to do this is to reduce the selection pressure by using chemicals as seldom as possible, and in particular, care should be taken not to spray populations with a compound to which resistance is already present.

Resistance to pyrethroids, carbamates, organophosphates and chlorinated hydrocarbons is well established in *M. persicae*, but in order to manage this resistance the occurrence and relationship between these mechanisms needs to be elucidated and methods for quickly and easily determining which combination of resistance mechanisms, if any, is present in a particular population are required.

5.3 Diagnostics

In order to understand the extent and spread of resistance and its dynamics in field populations it is vital to be able to accurately detect resistance in individual aphids. If these data are to be used to select appropriate control measures they need to be quickly available. With this in mind a PCR-based assay for the kdr and super-kdr mutations has been developed to run alongside existing resistance detection methodology. The existing tests for MACE and elevated esterases are already used to inform industry and growers, via a weekly resistance bulletin and popular magazine articles (Harrington *et al.*, 2003), providing interested parties with information on the resistance levels within the UK *M. persicae* population (and the virus load) and preventing unnecessary spraying that increases the selection and spread of resistant individuals and wastes money.

5.4 Spread of insecticide resistance

It is clear from the field and trap data collected that insecticide resistance is well established in *M. persicae* populations. In particular elevated levels of esterase and the kdr mutation are distributed widely. The most common kdr/super-kdr genotypes are SS/SS and SR/SS. The kdr heterozygote does have some advantage over the susceptible homozygote at field rate under simulated field conditions in the laboratory (Foster *et al.*, 2002a), although it is not as resistant as the resistant homozygote. Both super-kdr heterozygotes and resistant homozygotes are effectively immune to pyrethroids in all laboratory bioassays conducted up to 1000 ppm (which is well above field rate), this means the effective dominance will be very close to 1 at field rate and in fact at all rates tested (Bourguet *et al.*, 2000).

The global spread of these resistance mutations is probably due to both aphid migration and gene flow, and to the likely multiple origins of these resistance mechanisms (Anstead *et al.*, 2005). Data presented in this thesis show there has been historical gene-flow between Northern Europe, Southern Europe and Australasia. This conclusion is supported by work from Australia (Wilson *et al.*, 2002) using microsatellites showing *M. persicae* in Australia are probably descended from a moderate number of European migrants.

5.5 Interactions of mechanisms

One of the most interesting discoveries from this research was that a number of *M. persicae* individuals from throughout Europe possessed all three resistance mechanisms under consideration; R3 elevated carboxylesterase, the MACE mutation and both the kdr and super-kdr mutations. These extreme individuals would resist treatment with any organophosphates, pyrethroids and di-methyl carbamates. There was a widespread tendency for mechanisms to associate with each other. Elevated esterase and kdr and super-kdr were all more likely to occur in the same individual than would be expected by chance. This is perhaps not surprising as both mechanisms confer resistance to pyrethroids. MACE also showed an association with both esterase and the kdr mutations. This association is probably caused by insecticide selection. Some commercial insecticide treatments contain a mixture of carbamates and pyrethroids (e.g. a mix of pirimicarb and lambda-cyhalothrin) and in other cases such compounds are co-applied as tank- mixes. This would select strongly for aphids with resistance to both compounds. In a similar way during a season successive sprays of different compounds on the same asexual aphid lineages will have the same effect. This would be even more obvious in areas where sexual recombination is rare or absent as this effect will persist and be strengthened from year to year. These data showed that this was indeed the case as the association between MACE and the kdr mutations was strongest in Northern Europe. In fact selection of this kind is one reason why clonal lineages can be selectively advantageous over the short/medium term. Such an advantage has been shown in Scottish populations of *M. persicae* with the MACE mutation (Fenton *et al.*, 2005). During spraying, these asexually reproducing MACE aphids increase in numbers to dominate populations on treated crops, numbers fall again after spraying ceases due presumably to a selective disadvantage in the absence of selection insecticides, but enough survive for the same process to happen in the subsequent season.

5.6 Fitness costs

There has been a lot of work looking at the costs of insecticide resistance in insects including mosquitoes (Bourguet *et al.*, 2004) and *M. persicae* (Foster *et al.*, 1997; Foster *et al.*, 1999). Whilst fitness costs were not addressed directly in this study, some interesting inferences can be made from data collected.

Strong heterozygote excess was found for both the kdr and the super-kdr mutations. In a single population this could simply have been caused by the over-representation of a single highly successful clone or clones that happened to be heterozygous for these mutations. However the presence of this pattern in practically all samples indicates this is a real effect. In particular, homozygous resistant individuals seem to have an added fitness cost. What cannot be determined is whether this is due to the resistance mutations themselves or is due to other associated deleterious recessive mutations. If non-resistance genes were to blame, this fitness cost might be expected to slowly decrease over time as recombination separates the resistance mutations from them.

In the UK, *M. persicae* with kdr were found during every month in the suction trap samples. There was no evidence of significant selection against the kdr mechanism at any point during the year. Over-wintering survival was also good, with more kdr insects found in the spring of 2003 than the autumn of 2002, this over-wintering increase in kdr was consistent with over-wintering in the egg stage in France (Guillemaud *et al.*, 2003b), where an increase in kdr was also found. The super-kdr mutation however shows a very different pattern of distribution, reaching a frequency of 0.4 on occasions but quickly disappearing in subsequent months. This sudden rise probably indicated selection by spraying and the fall that followed occurred as it was out-competed by other clones in the absence of insecticides. This fitness cost, is not however strong enough to remove the resistance mutation completely, as it has been found every year since, although its distribution is still typically patchy and short-lived.

5.7 Future work

It is clear from any study of resistance that constant vigilance is needed to prevent control failures due to insecticide resistance. *M. persicae* has shown an innate ability to develop resistance to insecticides. As new chemistries become available early detection of any resistance is vital. Over the last few years growers are starting to use new compounds to control *M. persicae* and other aphids. Neonicotinoids are becoming increasingly important in the control of aphids and other insects. Resistance to neonicotinoids has been reported in a number of insect pests (Nauen and Denholm, 2005). So far no strong resistance has been found in *M. persicae* although there is an intrinsic level of

tolerance in some clones (Foster *et al.*, 2003a). Pymetrozine, an anti-feedant compound, is also becoming more widely used and there are no reports of resistance so far. Tests of various aphid lines showed no resistance or tolerance in *M. persicae* (Foster *et al.*, 2002b). These new chemistries are especially important when MACE or kdr mutations are present. Their increased use is likely to lead to resistance problems sooner rather than later so monitoring is an essential part of continuing work.

In regards to pyrethroid resistance there are fundamental questions that remain to be answered. One intriguing finding involves the dominance of kdr and super-kdr. Kdr is almost completely recessive in most insects (Plapp *et al.*, 1976; Liu *et al.*, 1981; Halliday and Georghiou, 1985; Payne *et al.*, 1988; Ru *et al.*, 1998). As might be predicted as only 0.1% of channels need to be affected to disrupt nerve function (Narahashi, 1992). In a heterozygote both alleles should be transcribed and translated. This would mean half the sodium channels produced would be resistant and half susceptible. In *M. persicae* however kdr and super-kdr heterozygotes both give some protection from pyrethroid applications in bioassay and simulator experiments (Eleftherianos, 2003; Foster *et al.*, 2002b). Neither kdr nor super-kdr is close to recessive, in fact super-kdr is effectively dominant at field rates of deltamethrin and lambda-cyhalothrin (Eleftherianos, 2003). This implies that in a heterozygote, susceptible-type sodium channels are not being formed. Previous work has shown that both susceptible and resistant type mRNA is found in equal proportions (Martin Williamson pers. comm). It is therefore possible that some sort of post-transcriptional control is involved. Once such a mechanism had arisen it could have a selective advantage over super-kdr homozygous resistant aphids which suffer an added fitness cost from their homozygosity.

The nature of the fitness costs associated with resistance is also not fully understood. Some studies have shown fitness costs associated with insecticide resistance in *M. persicae*. Elevated esterase levels were associated with reduced movement from senescing leaves (Foster *et al.*, 2003c; Foster *et al.*, 1997) and MACE was associated with a slower rate of reproduction (Foster *et al.*, 2003b). The presence of the kdr mutation in *M. persicae* clones was associated with a reduced response to alarm pheromone (Foster *et al.*, 2003c; Foster *et al.*, 1999). Further experiments included a heterozygous super-kdr clone, which showed the lowest response to alarm pheromone of any clone

(Eleftherianos, 2003). Given the apparent very high selection against super-kdr homozygous resistant individuals it would be interesting to see if they have an even lower response to alarm pheromone and subsequent higher fitness cost and to see if there are any other fitness costs associated with this mutation.

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High-throughput detection of knockdown resistance in *Myzus persicae* using allelic discriminating quantitative PCR

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Evidence for multiple origins of identical insecticide resistance mutations in the aphid *Myzus persicae*

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